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AUTHOR(S):

Yamasoba, Daichi; Sato, Kei; Ichinose, Takuya; Imamura, Tomoko; Koepke, Lennart; Joas, Simone; Reith, Elisabeth; ... Sauter, Daniel; Koyanagi, Yoshio; Takeuchi, Osamu

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N4BP1 restricts HIV-1 and its inactivation by MALT1 promotes viral reactivation

Daichi Yamasoba^{1,2,5}, Kei Sato^{3,6,7}, Takuya Ichinose^{1,2,5}, Tomoko Imamura², Lennart Koepke⁸, Simone Joas⁸, Elisabeth Reith⁸, Dominik Hotter⁸, Naoko Misawa³, Kotaro Akaki^{1,2,5}, Takuya Uehata^{1,2}, Takashi Mino^{1,2}, Sho Miyamoto⁴, Takeshi Noda⁴, Akio Yamashita⁹, Daron M. Standley¹⁰, Frank Kirchhoff⁸, Daniel Sauter⁸, Yoshio Koyanagi³ and Osamu Takeuchi^{1,2*}

¹Department of Medical Chemistry, Graduate School of Medicine, ²Laboratory of Infection and Prevention, ³Laboratory of Systems Virology, ⁴Laboratory of Ultrastructural Virology, Institute for Frontier Life and Medical Sciences, ⁵Graduate School of Biostudies, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

⁶CREST, Japan Science and Technology Agency, Saitama 322-0012, Japan.

⁷Department of Systems Virology, Institute for Medical Science, the University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

⁸Institute of Molecular Virology, Ulm University Medical Center, 89081 Ulm, Germany

⁹Department of Molecular Biology, Yokohama City University School of Medicine, Kanagawa 236-0004, Japan

¹⁰Department of Genome Informatics, Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, 3 Yamada-oka, Suita, Osaka 565-0871, Japan

*e-mail: otake@mfour.med.kyoto-u.ac.jp.

*D. Yamasoba et al.***Abstract**

RNA modulating factors not only regulate multiple steps of cellular RNA metabolism, but also emerge as key effectors of the immune response against invading viral pathogens including human immunodeficiency virus type-1 (HIV-1). However, cellular RNA binding proteins involved in the establishment and maintenance of latent HIV-1 reservoirs have not been extensively studied. Here, we screened a panel of 62 cellular RNA binding proteins and identified NEDD4 binding protein 1 (N4BP1) as potent interferon-inducible inhibitor of HIV-1 in primary T cells and macrophages. N4BP1 harbors a prototypical PIN-like RNase domain and inhibits HIV-1 replication by interacting with and degrading viral mRNA species. Upon activation of CD4⁺ T cells, however, N4BP1 undergoes rapid cleavage at Arg509 by the paracaspase MALT1. Mutational analyses and knockout studies revealed that MALT1-mediated inactivation of N4BP1 facilitates the reactivation of latent HIV-1 proviruses. Taken together, our findings demonstrate that the RNase N4BP1 is an efficient restriction factor of HIV-1 and suggest that inactivation of N4BP1 by induction of MALT1 activation might facilitate elimination of latent HIV-1 reservoirs.

44 **Introduction**

45 Host cells are equipped with sophisticated mechanisms to prevent or inhibit viral
46 infection. HIV-1 replication, for example, is targeted by a plethora of restriction factors
47 including APOBEC3, TRIM5 α , Tetherin, SAMHD1, GBP5 and MX2¹⁻¹⁰. Although
48 these factors may suppress HIV-1 replication at various steps through multiple
49 independent mechanisms, they are usually counteracted or evaded by the virus. Another
50 characteristic shared by many host restriction factors is their inducibility by type I
51 interferons (IFNs), which play a pivotal role in the host defense against viral infection
52^{11,12}.

53 In search of novel effective antiretroviral mechanisms, nucleic acid binding
54 proteins are of particular interest since they are not only important regulators of antiviral
55 gene expression, but may also act as direct effectors of the antiviral immune response
56^{13,14}. Furthermore, they may modulate sensing of viral infection by binding to and/or
57 degrading viral RNA or DNA species or by directly acting as pattern recognition
58 receptors. One example of a nucleic acid-binding protein with antiviral activity is the
59 zinc-finger antiviral protein (ZAP). Initially, ZAP was shown to degrade mouse leukemia
60 virus (MLV) RNA by recruiting the exosome complex^{15,16}. ZAP recognizes RNA
61 sequences enriched in CG dinucleotides, which are suppressed in the genomes of HIV-1
62 and many other vertebrate viruses¹⁷. As a result, HIV-1 engineered to contain a higher
63 number of CG dinucleotides is more sensitive to ZAP than the respective parental virus.
64 Another example of an antiviral nucleic acid binding protein is SAMHD1. This dNTP
65 triphosphohydrolase blocks HIV-1 reverse transcription in myeloid cells and resting T
66 cells by depleting cellular dNTP pools^{9,10,18}. Finally, the endoribonuclease Regnase-1
67 (also known as MCP1P1) has been suggested to degrade retroviral mRNAs¹⁹. Given the
68 large number of human RNA binding proteins identified in recent studies^{20,21}, it seems
69 highly likely that additional RNA binding proteins with key roles in antiviral immunity
70 remain to be discovered. As the example of SAMHD1 illustrates^{22,23}, some of these

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71 restriction factors might be preferentially active in resting CD4⁺ T cells and play a role in
72 the establishment and maintenance of latent HIV-1 reservoirs.

73 To discover novel restriction factors targeting viral RNA, we screened a
74 collection of cellular proteins containing various RNA binding domains for
75 antiretroviral activity.

76 **Results**

77 **Identification of N4BP1 as a host factor inhibiting HIV-1**

78 To identify as-yet-unknown host restriction factors suppressing HIV-1 replication by
79 binding to viral RNA, we selected 62 expression plasmids from mammalian gene
80 collection (MGC) clones encoding proteins harboring at least one RNA binding domain,
81 such as CCCH- or CCHC-type Zinc Fingers (ZF), KH domains or RNase folds (see
82 **Supplementary Table 1**), since proteins harboring these domains may be involved in the
83 suppression of HIV-1^{17,19}. We co-transfected HEK293T cells with the infectious HIV-1
84 NL4-3 molecular clone and the 62 expression plasmids, and determined infectious HIV-1
85 yield in the culture supernatants by infecting TZM-bl indicator cells 48 hours
86 post-transfection. Of the 62 proteins analyzed, NEDD4 binding protein 1 (N4BP1) was
87 the most potent inhibitor, decreasing infectious HIV-1 NL4-3 production by >20-fold
88 (**Fig. 1a**). Immunoblot analysis of cells producing HIV-1 NL4-3 or the primary HIV-1
89 isolate AD17 revealed that N4BP1 expression decreased the expression of viral Env and
90 Gag proteins in a dose-dependent manner (**Fig. 1b**). Analyzing a broader panel of
91 primate lentiviruses, including transmitted/founder (TF) and chronic control (CC)
92 HIV-1 strains, we found that N4BP1 reduced infectious yield of all HIV-1, HIV-2 and
93 SIVcpz strains examined (**Fig. 1c and Supplementary Fig. 1**). Thus, N4BP1 is a broad
94 and potent inhibitor of evolutionarily diverse primate lentiviruses.

96 **N4BP1 is IFN-inducible and restricts HIV-1 infection in human T cells**

97 Since antiretroviral host restriction factors are frequently inducible by IFN and virus
98 infection, we examined if the expression of N4BP1 is IFN-inducible in T cells. IFN- α
99 stimulation of Jurkat T cells increased N4BP1 expression at both the mRNA and protein
100 level (**Fig. 2a and 2b**). Similarly, *N4BP1* expression was induced about 3-fold by IFN- α
101 stimulation in CD4⁺ T cells from healthy human donors (**Fig. 2c**). Notably, all 12 human
102 IFN- α subtypes, but not IL-27, increased N4BP1 protein levels by about 2- to 4-fold in

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primary CD4⁺ T cells (**Supplementary Fig. 2a and 2b**). Furthermore, HIV-1 NL4-3 infection induced the expression of mRNAs encoding IFN- β , N4BP1 and the IFN-inducible proteins Tetherin/BST-2 and ISG15 in Jurkat cells (**Fig. 2d**). N4BP1 gene expression was also significantly upregulated in the spleens of HIV-1 infected humanized mice (**Fig. 2e**). Collectively, these data show that N4BP1 is a type I IFN- and HIV-1-inducible protein with potent anti-HIV-1 activity.

To determine whether endogenous N4BP1 restricts HIV-1 in human T cells, we used the CRISPR/Cas9 system to generate seven Jurkat cell lines lacking N4BP1 expression (**Fig. 2f, left panel**). HIV-1 replicated with faster kinetics in all seven *N4BP1*-deficient (KO) cell lines compared to Cas9-expressing control cells in response to HIV-1 infection (MOI 0.01) (**Fig. 2f, right panel**). Reconstitution of N4BP1 expression via a doxycycline-inducible Tet-on system (**Fig. 2g, left panel**) rescued inhibition of HIV-1 replication (**Fig. 2g, right panel**). Noteworthy, growth, apoptosis rates, or global protein synthesis were not altered between control and N4BP1 KO Jurkat cells without HIV-1 infection (**Supplementary Fig. 3a-3c**). Furthermore, N4BP1 deficiency did not affect the expression of a set of host genes such as *IFNB* and *NFKBIA* (**Supplementary Fig. 4a**). Consistently, the expression of I κ B α , Tubulin- α and GAPDH proteins was not different between control and *N4BP1* KO Jurkat cells (**Supplementary Fig. 4b**), suggesting that the anti-HIV-1 effect of N4BP1 is direct and not mediated by the regulation of host genes. In agreement with the results of N4BP1 KO cells, siRNA-mediated knockdown of N4BP1 in Jurkat cells (**Supplementary Fig. 5a**) resulted in a marked increase in viral RNA expression 72 h after infection with HIV-1 NL4-3, without significantly affecting *IFNB* expression levels (**Supplementary Fig. 5b and 5c**).

Conversely, we also generated four Jurkat cell clones stably over-expressing N4BP1 or a control vector (**Fig. 2h, left panel**). N4BP1 over-expression fully prevented HIV-1 replication at low MOI (0.001) (**Fig. 2h, central panel**). Even at higher MOI (0.01), N4BP1 over-expression prevented or substantially delayed HIV-1 replication (**Fig.**

130 **2h, right panel**). Furthermore, the expression of viral RNAs including *tet/rev*, *vif* and *gag*
131 was suppressed in N4BP1 over-expressing Jurkat cells (**Supplementary Fig. 5d and 5e**).
132 These results demonstrate that N4BP1 restricts HIV-1 replication in human T cells.

133 Since two out of four N4BP1 overexpressing Jurkat cell clones allowed delayed
134 but detectable replication of HIV-1 (**Fig. 2h**), we examined whether N4BP1 expression
135 was altered in these cells following HIV-1 infection. To avoid artifacts due to differences
136 in viral replication and spread of infection, we used an *env*-deficient (Δenv)
137 VSV-G-pseudotyped HIV-1 NL4-3 construct (**Supplementary Fig. 5f**). Consistent with
138 the results obtained with replication-competent HIV-1, production of Gag was suppressed
139 in N4BP1 overexpressing cells compared to control cells in single cycle infection.
140 Notably, however, N4BP1-mediated inhibition of Gag expression was less pronounced at
141 higher MOI, suggesting that high amounts of HIV-1 may saturate the inhibitory effect of
142 N4BP1. Furthermore, HIV-1 infection did not reduce N4BP1 expression levels
143 (**Supplementary Fig. 5f**).

144

145 **N4BP1 restricts HIV-1 in primary macrophages**

146 IFN- α induced N4BP1 mRNA and protein expression not only in T cells (**Fig. 2a-2c**) but
147 also in THP-1-derived macrophage-like cells (**Fig. 3a and 3b**). In addition, we found that
148 N4BP1 is constitutively expressed in primary human monocyte-derived macrophages
149 (MDMs) and further upregulated by IFN- α stimulation (**Fig. 3c-3e**). To examine the
150 antiretroviral activity of N4BP1 in primary macrophages, we knocked down N4BP1
151 using three different siRNAs (**Fig. 3f**) before infecting the cells with the
152 macrophage-tropic HIV-1 strain AD8. The three N4BP1-specific siRNAs increased
153 infectious HIV-1 AD8 yield by 6.5-, 10.8- and 2.6-fold, respectively, at day 3 and by
154 16.2-, 14.8- and 4.3-fold by day 6 post-infection (**Fig. 3g**). Thus, N4BP1 restricts HIV-1
155 in both CD4⁺ T cells and macrophages.

156

157 N4BP1 degrades HIV-1 RNA

158 To decipher the molecular mechanisms underlying the antiviral effect of N4BP1, we
159 investigated whether its inhibitory activity is limited to HIV-1 and related primate
160 lentiviruses. Overexpression of N4BP1 in HEK293T cells also inhibited MLV; genus
161 *Gammaretrovirus*) and human foamy virus (HFV; genus *Spumavirus*) (**Fig. 4a**). However,
162 N4BP1 failed to suppress influenza A virus mRNA expression in HEK293T cells
163 (**Supplementary Fig. 6a**). Furthermore, N4BP1 overexpression did not reduce
164 production of infectious influenza A virus in HEK293T cells (**Supplementary Fig. 6b**),
165 arguing against a general and unspecific effect of N4BP1 on viral RNA expression.

166 Since N4BP1 reduces both HIV-1 mRNA and protein expression levels, we
167 analyzed whether it affects proviral transcription via the long terminal repeat (LTR) using
168 a reporter system expressing luciferase under the control of the viral LTR promoter.
169 While NL4-3 Δenv induced reporter gene expression, co-expression of N4BP1 failed to
170 suppress HIV-1 LTR-mediated gene expression (**Supplementary Fig. 7a**). In contrast,
171 N4BP1 suppressed HIV-1 expressed under the control of the CMV promoter (pCMV259),
172 but did not affect EGFP expression from the same promoter (**Supplementary Fig. 7b**
173 **and 7c**). Together, these results suggest that N4BP1 suppresses HIV-1, but not EGFP, at a
174 post-transcriptional level.

175 N4BP1 was originally identified as a target of the E3 ubiquitin ligase NEDD4,
176 resulting in its proteasomal degradation in the nucleolus and promyelocytic leukemia
177 (PML) bodies^{24,25}. N4BP1 harbors a potential nuclease domain in addition to two KH
178 domains, which represent canonical single-stranded nucleic acid binding domains (**Fig.**
179 **4b**)²⁶. The nuclease domain of N4BP1 is highly conserved among mammalian species
180 and structurally predicted to form a catalytic pocket with conserved aspartic acids (**Fig.**
181 **4c**). Using a subgenomic sequence of HIV-1 NL4-3 as substrate, we found that
182 recombinant N4BP1 degrades viral RNA *in vitro* (**Fig. 4d and Supplementary Fig. 8a**).
183 Structural modeling of the human N4BP1 RNase domain revealed that it harbors a

184 catalytic center whose structure and primary amino acid sequence is similar to that of
185 Regnase-1 (**Fig. 4e and Supplementary Fig. 8b**). A point mutation of Asp623, which is
186 predicted to be essential part of the catalytic center, to Asn (D623N) fully abrogated the
187 RNase activity of N4BP1 (**Fig. 4d**). Importantly, structural modeling suggests that the
188 D623N mutation does not alter the overall structure of the RNase domain of N4BP1 (**Fig.**
189 **4e**).

190 Consistently, Northern blot analysis showed that N4BP1 reduces un-, singly-
191 and multi-spliced viral mRNAs in an RNase activity-dependent manner, while ribosomal
192 RNA was not affected (**Fig. 4f**). In contrast, incoming viral RNA was not degraded as
193 knockdown of N4BP1 did not significantly alter the amount of early or late reverse
194 transcriptase (RT) products or the amount of integrated proviral DNA in infected Jurkat
195 cells (**Supplementary Fig. 8c**).

196 To test whether N4BP1 directly binds HIV-1 RNA, we performed an RNA
197 immunoprecipitation (RIP) assay using the N4BP1 D623N mutant, in which the
198 RNA-protein interaction is expected to be stable due to the lack of RNase activity. The
199 RIP-qPCR assays revealed that the N4BP1 D623N mutant binds several HIV-1 mRNA
200 species including splice products expressing Tat/Rev, Vif and Gag (**Fig. 4g**). In agreement
201 with RNase-dependent restriction, wild-type N4BP1, but not the catalytically inactive
202 mutant D623N suppressed viral protein expression (**Fig. 4h and Supplementary Fig.**
203 **8d**) and infectious HIV-1 yield (**Fig. 4i**) without affecting cell viability (**Supplementary**
204 **Fig. 8e and 8f**). Collectively, these data demonstrate that N4BP1 restricts HIV-1
205 replication by binding and degrading viral mRNA species.

206

207 **MALT1 degrades N4BP1 in T cells upon TCR-mediated activation.**

208 Antiretroviral restriction factors are often counteracted by HIV-1 accessory proteins.
209 N4BP1, however, inhibited wild-type HIV-1 NL4-3 and a mutant lacking all four
210 accessory proteins (NL4-3 $\Delta 4$) with similar efficiencies (**Supplementary Fig. 9**). Thus,

we next examined N4BP1 expression levels in activated CD4⁺ T cells that are highly permissive for HIV-1 replication and resting T cells thought to represent the main reservoir of latent HIV-1. Interestingly, N4BP1 protein levels were drastically decreased in primary CD4⁺ T cells activated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin or by treatment with anti-CD3/CD28 antibodies, but not with IFN- γ or IL-2 (**Fig. 5a**). Kinetic analysis revealed that N4BP1 levels started to decrease at 0.5 h after PMA/ionomycin stimulation and remained undetectable for at least 8 hours (**Fig. 5b**). Notably, N4BP1 mRNA levels were not markedly affected by PMA/ionomycin treatment (**Supplementary Fig. 10a**). In support of N4BP1 protein degradation, a ~72 kDa cleavage product appeared in PMA/ionomycin-treated cells (**Fig. 5b**) just above non-specific bands (65 kD and 40 kD) which is also present in N4BP1 KO cells (**Fig. 2f**). Although N4BP1 was reported to undergo NEDD4-mediated polyubiquitination²⁵, proteasome inhibitor treatment did not prevent PMA/ionomycin-mediated degradation of N4BP1 (**Supplementary Fig. 10b**). Further experiments revealed that PMA alone, but not ionomycin, is sufficient to decrease N4BP1 levels (**Supplementary Fig. 10c**). Similar to T cell receptor (TCR) signals, PMA activates signaling pathways via the CARMA1-BCL10-MALT1 signalosome²⁷. MALT1 is known to cleave RNA binding proteins Regnase-1 and Roquin proteins^{28,29}. Consistent with a previous report, overexpression of Regnase-1 also suppressed infectious HIV-1 NL4-3 production, whereas expression Roquin-1 or -2 had no inhibitory effect (**Supplementary Fig. 10d**). We therefore hypothesized that N4BP1 might also be cleaved by MALT1. Indeed, knockout of MALT1 in Jurkat cells abrogated degradation of N4BP1 in response to stimulation (**Fig. 5c**). Furthermore, the MALT1 inhibitor zVRPR-fmk, but not a pan-caspase inhibitor zVAD-fmk, suppressed cleavage of N4BP1 (**Fig. 5d**). Thus, the protease activity of MALT1 is essential for N4BP1 cleavage upon T cell activation.

MALT1 cleaves N4BP1 at R509

238 The appearance of a ~72 kDa cleavage product (**Fig. 5b**) suggested that the MALT1
239 cleavage site(s) is located in the central region of N4BP1, between the KH and RNase
240 domains (**Fig. 5e**). Thus, we generated a series of C-terminally truncated variants to
241 determine the exact position of the cleavage site (**Fig. 5e**). Immunoblot analysis
242 revealed that the electrophoretic mobility of an N4BP1 mutant comprising the
243 N-terminal 500 amino acids was close to that of MALT1 cleaved N4BP1 (**Fig. 5f**).
244 MALT1 specifically cleaves after arginine residues ²⁷, and analysis of previously
245 identified MALT1 substrates revealed a putative [S/P]-R-G consensus target sequence
246 for this protease (**Fig. 5g and Supplementary Fig. 10e**). N4BP1 harbors a highly
247 conserved SRG motif at positions 508-510 (**Fig. 5h**), and R509A mutant N4BP1 was
248 not cleaved by MALT1 expressed together with BCL10 (**Fig. 5i**). Furthermore,
249 PMA/ionomycin stimulation of Jurkat cells induced cleavage of WT Flag-N4BP1, while
250 the Flag-N4BP1 R509A mutant was resistant to cleavage (**Fig. 5j**). Notably, mutation of
251 R509A rendered N4BP1 resistant to MALT1 cleavage without impairing its antiviral
252 activity (**Fig. 5k**).

253

254 **Degradation of N4BP1 promotes reactivation of latent HIV-1**

255 Degradation of N4BP1 by MALT1 might be involved in the reactivation of latent HIV-1
256 by T cell activation. To investigate this possibility, we first examined N4BP1 expression
257 in three different human T cell lines harboring latent HIV-1 proviruses ³⁰⁻³². N4BP1 was
258 constitutively expressed and downmodulated upon PMA stimulation in all cell lines
259 tested (**Supplementary Fig. 11a-c**). As expected, PMA-induced degradation of N4BP1
260 was abrogated in MALT1 knockout JNLGFP, J-Lat10.6 and J-Lat5A8 cells (**Fig. 6a, 6b,**
261 **Supplementary Fig. 11d**). Thus, MALT1 is required for PMA-induced degradation of
262 N4BP1 in latently HIV-1 infected T cells. MALT1-knockout JNLGFP, J-Lat10.6 and
263 J-Lat5A8 cells showed significantly lower levels of HIV-1 reactivation than control
264 cells in response to PMA stimulation (**Fig. 6c, 6d, and Supplementary Fig. 11e**). After

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265 induced expression of N4BP1 in J-Lat10.6 and JNLGFP cells by a Tet-On system,
266 wild-type but not R509A N4BP1 was degraded in response to PMA stimulation (**Fig. 6e**
267 **and 6f**). Intriguingly, the MALT1-resistant N4BP1 R509A mutant suppressed the
268 reactivation of J-Lat10.6 and JNLGFP cells more efficiently than WT N4BP1 as
269 examined by the changes in HIV-1 p24 levels in the culture supernatants (**Fig. 6e and**
270 **6f**). We further established N4BP1 KO JNLGFP cells (**Fig. 6g**), and reconstituted them
271 with WT N4BP1 or the R509A mutant thereof using the inducible Tet-On system (**Fig.**
272 **6h**). Consistent with the overexpression data, JNLGFP cells reconstituted with R509A
273 N4BP1 potently suppressed reactivation of HIV-1, whereas cells reconstituted with
274 wild-type N4BP1 failed to do so. (**Fig. 6h and 6i**). Thus, inactivation of N4BP1 by
275 MALT1 supports latency reversal upon activation of latently HIV-1 infected T cells
276 (**Supplementary Fig. S12**).

277

278 **Discussion**

279 Our study demonstrates that HIV-1 latency and reactivation are controlled by N4BP1 at
280 the post-transcriptional level. Notably, two mechanisms may contribute to the
281 maintenance of viral latency by N4BP1: First, N4BP1 generally degrades spliced- and
282 un-spliced HIV-1 transcripts, thereby preventing their translation into viral proteins and
283 progeny virion formation. Second, N4BP1 may continuously keep HIV-1 Tat levels
284 below the threshold required for reactivation and efficient transcription of viral genes.
285 Intriguingly, N4BP1 is rapidly inactivated by MALT1 in response to stimuli inducing
286 HIV-1 reactivation, indicating that changes in MALT1 activation levels regulate HIV-1
287 reactivation. Thus, manipulation of MALT1 activity may be a promising approach to
288 control HIV-1 latency and reactivation. Notably, treatment with the MALT1 inhibitor
289 MI-2 has previously been shown to induce death of latently HIV-1 infected cells, and
290 death rates were further increased by PMA stimulation ³³. However, specificity of
291 MALT1 inhibition and the MALT1 targets involved in this process remained unclear.
292 Thus, further studies are required to decipher the suitability of MALT1 as a target for
293 therapeutic intervention.

294 An important open question is how N4BP1 specifically recognizes retroviral
295 RNA, without degrading for example Influenza A virus or ribosomal RNAs. Given that
296 multi-spliced HIV-1 RNA (encoding Tat and Rev) as well as singly or un-spliced viral
297 mRNAs are degraded, N4BP1 might target motifs or structures in un-spliced viral mRNA,
298 the *tat/rev* encoding region or end modifications found in all three mRNA classes. The
299 frequency of CG dinucleotides is markedly suppressed in the genomes of HIV-1 as well
300 as other vertebrate viruses, thereby attenuating ZAP-mediated restriction ¹⁷. Given that
301 N4BP1 suppresses various lenti- and retro-viruses including HIV-1, sequence motif(s)
302 other than CG dinucleotides could be recognized by N4BP1.

303 N4BP1 is localized in the nucleus especially in the nucleolus and/or PML bodies
304 ²⁵. Intriguingly, HIV-1 transcripts are specifically re-localized into nucleoli for viral gene

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expression³⁴. In addition, latent HIV-1 was reported to colocalize with PML bodies, with PML binding to the latent HIV-1 LTR promoter³⁵. Degradation of PML led to the activation of viral transcription together with the release of histone methyltransferase G9a³⁵. Given that HIV-1 RNA is suppressed by N4BP1, it is tempting to speculate that N4BP1 co-transcriptionally degrades viral mRNA in PML bodies, thereby contributing to the maintenance of latency. Although MALT1 is majorly present in the cytoplasm, this protein harbors a nuclear export signal (NES), and is reported to shuttle between nucleus and cytoplasm³⁶. Thus, MALT1 might cleave N4BP1 in the nucleus.

Our data suggest that N4BP1 is not directly counteracted by HIV-1 accessory proteins. However, it is possible that HIV-1 has evolved means to evade N4BP1. Furthermore, expression of N4BP1 was diminished in T cells stimulated with PMA/ionomycin or via TCR-CD3/CD28 ligation. Considering that HIV-1 replicates efficiently in activated but not in quiescent CD4⁺ T cells, it is tempting to speculate that reduction of N4BP1 facilitates HIV-1 replication in activated T cells. We discovered that cleavage and inactivation of N4BP1 in T cells is mediated by the protease MALT1 and identified R509 as its cleavage site. Previously described MALT1 substrates include host mRNA regulators such as Regnase-1 and Roquin. These proteins are critical for controlling immune reactions as they destabilize host mRNAs encoding proinflammatory cytokines and proteins involved in T cell activation^{37,38}. It is intriguing to explore the function of N4BP1 in the control of immune responses and future studies will uncover the functional roles of N4BP1 in regulating host mRNAs *in vivo*.

A recent study determining type I interferomes of fibroblasts in multiple vertebrate species identified 62 evolutionarily conserved interferon-stimulated genes (ISGs)¹². Interestingly, N4BP1 was also among these core ISGs. Upon IFN- α stimulation, N4BP1 suppresses HIV-1 infection by inducing its expression and/or altering its RNase activity. Additionally, the MALT1-cleavage site as well as the RNase

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332 domain are conserved among the N4BP1 orthologs of different mammalian species,
333 consistent with IFN-inducibility. These notions imply that the function of N4BP1 in
334 antiviral immunity is conserved among different species. Given that human N4BP1
335 suppresses a variety of retroviruses, but not influenza A virus, it is tempting to speculate
336 that N4BP1 might have an ancestral function in specifically controlling retroviral
337 infection. Furthermore, it will be interesting to explore whether N4BP1 is involved in
338 the post-transcriptional silencing of endogenous retroviruses.

339 In this study, we identified N4BP1 as a HIV-1 restriction factor by screening
340 proteins that harbor potential RNA binding domains. As our screening approach did not
341 include all RNA binding proteins, it is possible that additional anti-retroviral RNA
342 binding factors remain to be discovered. Furthermore, an over-expression-based
343 screening approach may fail to identify antiviral proteins that are endogenously
344 expressed to high levels, if over-expression does not further increase their abundance.
345 Therefore, further studies are required to elucidate the role of RNA binding proteins in
346 restriction of HIV-1, e.g. by using more complete sets of RNA binding proteins and/or
347 loss of function screening systems.

348 In summary, we identified N4BP1 as an RNase that functions as an antiretroviral
349 restriction factor by degrading various HIV-1 mRNA species. Although further studies
350 are required to precisely define the molecular mechanisms underlying target RNA
351 recognition by N4BP1, our findings clearly demonstrate that N4BP1 is a potent effector
352 of type I IFN-mediated anti-HIV-1 activity.

353

354 **Methods**

355 **Cell culture, Proviral Constructs and Transfection.**

356 Jurkat cells and THP-1 cells were obtained from the ATCC and grown in RPMI-1640
357 medium (Nacalai Tesque) supplemented with 10% fetal calf serum (FCS) and 50 μ M
358 β -mercaptoethanol (Nacalai Tesque). HEK293T cells were obtained from ATCC and
359 grown in DMEM supplemented with 10% fetal calf serum (FCS). TZM-bl cells and
360 J-Lat10.6 cells were obtained from the NIH AIDS Research and Reference Reagent
361 Program and maintained in DMEM medium (Nacalai tesque) supplemented with 10%
362 fetal calf serum (FCS) or in RPMI-1640 medium (Nacalai Tesque) supplemented with
363 10% fetal calf serum (FCS) and 50 μ M β -mercaptoethanol (Nacalai Tesque) respectively.
364 J-Lat5A8 cells were kindly provided by Warner C. Greene from the Gladstone Institute of
365 Virology and cultured in RPMI-1640 supplemented with 10% fetal calf serum. JNLGFP
366 cells were kindly provided by David N. Levy from the New York University College of
367 Dentistry and cultured in RPMI1640 supplemented with 10% of FCS. Cell lines were not
368 validated further or tested for micoplasma in our laboratory. Plasmid transfection
369 experiments for HEK293T cells were performed using Polyethylenimine Max Mw 40000
370 (polysciences). Plasmid transfection experiments for Jurkat cells were performed using
371 Neon Transfection System (Invitrogen) according to the manufacturer's protocol.
372 HEK293T cells were transfected 24 h after seeding in 12-well plates at a confluence of
373 70%. Cells and supernatants were harvested 48 hours post-transfection. The
374 pCMV-SPORT6 expression plasmids used for the screening of genes encoding proteins
375 potentially harboring RNA binding domains listed in **Supplementary Table 1** represent
376 verified full-length cDNA Clones (Open Biosystems) obtained through the Mammalian
377 Gene Collection (MGC). Regnase-1 and Roquin-1 expression plasmids have been
378 described previously³⁹. The Roquin-2 expression plasmid was kindly provided by Dr.
379 Hidenori Ichijo (The University of Tokyo). Replication competent HIV-1 particles were
380 obtained by transfecting HEK293T cells with the following HIV-1 infectious plasmids:

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381 pNL4-3 (cat#114)⁴⁰ was obtained through the NIH AIDS Reagent Program. HIV-1
382 clones pCH058 (cat#11856)⁴¹ and pAD17 (cat#12423)⁴² were obtained through the
383 NIH AIDS Reagent Program from Dr. Beatrice Hahn (University of Pennsylvania). The
384 SIVcpz molecular clone MB897⁴³, as well as HIV-1 pCH058 (6-month), pCH077
385 (6-month), pCH440, pCH200v2, pCH534 and CH042 were kindly provided by Beatrice
386 Hahn⁴⁴⁻⁴⁶. The HIV-1 AD8 infectious molecular clone was obtained from Cathleen
387 Collins (UCSD, San Diego) and has been described previously⁴⁷. The Moloney
388 Leukemia Virus strain pMLV48 was kindly provided by Dr. Komano Atsushi (Nagoya
389 Medical Center). The Human Foamy virus strain HSRV13 was kindly provided by David
390 Russel (University of Washington). pCMV259 was kindly provided by Junichi Sakuragi
391 (Osaka University)⁴⁸. Forty eight hours post-transfection, culture supernatants were
392 harvested, then filtrated, and stocked as a viral solution. For the replication assay, empty
393 vector or N4BP1 expressing Jurkat cells were seeded at 2×10^5 cells in 48 well plate.
394 Then, they were inoculated with HIV-1 NL4-3. Primary CD4⁺ T cells were isolated from
395 human blood from 3 healthy donors by Ficoll Paque gradient centrifugation and negative
396 selection using the RosetteSepTM Human CD4⁺ T cell Enrichment cocktail (Stem Cell
397 Technologies) and cultured in RPMI1640. Monocytes were separated from PBMCs by
398 plastic adherence and differentiated into monocyte-derived macrophages (MDM) using
399 AB-serum (10%) and macrophage colony stimulating factor (M-CSF, R&D Systems; 15
400 ng/ml). Primary human macrophages were differentiated from human peripheral blood
401 mononuclear cells from 3 healthy donors. PBMCs were seeded onto the plate in serum
402 free RPMI-1640 for 3 hours at 37 °C. Non-adherent cells in the supernatants were
403 discarded and adherent monocytes were cultured in RPMI-1640 with 10% FCS and
404 M-CSF (15 ng/ml, PeproTech) for 6 days.

405

406 **Generation of Virus Stocks**

407 HEK293T cells were sown in 6-well plates and transfected with proviral HIV-1 DNA (5

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408 μg) at a confluence of 70-80 % using a standard calcium phosphate transfection
409 protocol. For mock infection controls, HEK293T cells were treated with transfection
410 reagents only. Supernatants were harvested 40 h post transfection.

411

412 **Infectivity Assay**

413 Infectious HIV-1 released into the cell culture supernatant was quantified by infection of
414 TZM-bl reporter cells. Appropriate virus dilutions were added to 5×10^3 TZM-bl cells
415 per well of a 96-well plate. The cells were harvested 48 hours post-infection, and a
416 β -galactosidase assay was performed using the Galacto-Star Mammalian Reporter Gene
417 Assay System (Applied Biosystems) according to the manufacturer's protocol.
418 Galactosidase activity was quantified as relative light units per second (RLU/s) using a
419 1420 ALBOSX multilabel counter (Perkin Elmer).

420

421 **Plasmid Construction**

422 The cDNA of human N4BP1 was ligated into pFlag-CMV2 (Invitrogen), pEFs-Flag-SBP,
423 or CSII-CMV-MCS-IRES2-Bsd for mammalian cell expression. The site-directed mutant
424 expression vectors including pFlag-CMV2 N4BP1 D623N or R509A and pEFs-Flag-SBP
425 D623N were generated using the Quick change lighting Site-Directed Mutagenesis Kit
426 (Agilent). Deletion mutant cDNAs encoding amino acids 1-700 ($\Delta 1$), 1-600 ($\Delta 2$), 1-500
427 ($\Delta 3$) and 1-400 ($\Delta 4$) of N4BP1 were inserted into the pFlag-CMV2 vector. A lentiviral
428 packaging plasmid, CSII-CMV-MCS-IRES2-Bsd was provided by Dr. Miyoshi in Keio
429 University. N4BP1 and its R509A mutant were inserted into the pInducer20, a Tet-on
430 doxycycline-inducible lentiviral expression plasmid.

431

432 **Immunoblotting**

433 HEK293T cells were seeded in 12 well plates. Cell free viral solutions were pelleted by
434 ultracentrifugation of the culture supernatants at 40, 000 rpm for 1 hour at 4°C by using

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TL-100 (Beckman), and then lysed in the Immunoblot lysis buffer. Cell lysates were mixed with 3x loading sample buffer supplemented with 15% β -mercaptoethanol. Protein samples were resolved on a 5-20% NuPAGE gel (Invitrogen). Proteins were transferred from the SDS-PAGE gel to Immobilon-PVDF membranes (Merck Millipore). Proteins were labeled with antibodies against HIV-1 Env (1:2000; 16H3, Cat#12559, NIH AIDS Research and Reference Reagent program), p24 (1:2000, polyclonal; ViroStat), Vif (1:2000; #319; NIH AIDS Research and Reference Reagent program), Nef (1:2000; 3D12, ThermoFisher), Vpr (1:2000; 8D1, Cosmo Bio), Vpu (1:2000; #969, NIH AIDS Research and Reference Reagent program), Flag (1:1000; monoclonal; F7425, Sigma, or polyclonal; F7425, Sigma), Mouse IgG Isotype Control (1:1000; #31903, ThermoFisher); β -Actin (1:1000; polyclonal; sc-1615, Santa Cruz), MALT1 (1:1000; #2494, Cell Signaling Technology), GAPDH (1:1000; sc-47724, Santa Cruz), α -Tubulin (1:1000; T9026, Sigma), I κ B α (1:1000; C-21, Santa Cruz), GFP (1:1000; ab290, Abcam) and N4BP1 (1:2000, ab197079, Abcam). The anti-N4BP1 antibody recognizes the N-terminal portion of N4BP1 (aa 250-300).

450

451 **Drug Treatment**

Jurkat cells and THP-1 cells were stimulated with IFN- α (Sigma Aldrich, 1000 U/ml). Jurkat cells were stimulated with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA: Sigma Aldrich) with or without 500 nM ionomycin (Sigma Aldrich). Human primary CD4⁺ T cells were stimulated with IFN- α subtypes (50 ng/ml) (kindly provided by Kathrin Sutter, University Duisburg-Essen) or IL-27 (R&D Systems; Cat# 2526; 5 ng/ml) for 72 hours, IFN- γ (SIGMA Aldrich; 50 ng/ml), IL-2 (Miltenyi Biotech; 100 U/ml) or anti-CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28, Gibco) for 24 hours. JNLGFP, J-Lat5A8 and J-Lat10.6 cells were stimulated with 10-50 ng/ml PMA for 24 hours. zVRPR-fmk (Enzo Life Sciences), a MALT1 inhibitor, zVAD-fmk (R&D systems), a Pan-Caspase inhibitor and MG132 (Merck Millipore), proteasome inhibitor, were used

462 at concentrations of 100 μ M, 10 μ M and 0.001-10 μ M, respectively.

463

464 **RNA Isolation, RT and Quantitative RT-PCR**

465 Total RNA was isolated using Trizol reagent (Invitrogen). Reverse transcription was
466 performed using ReverTra Ace (TOYOBO) according to the manufacturer's instruction.

467 The relative RNA expression levels of *IFNB*, 18S, *N4BP1*, *tat/rev*, *vif*, *gag*, HIV-1 total
468 RNA, *BST2*, and *ISG15*, MLV *gag*, SIVcpz *gag*, HSRV13 *gag* and Influenza A segment 4
469 (HA) were measured by SYBR Green Real-Time PCR in Applied Biosystems Step One
470 Plus. Viral RNA in supernatants was extracted using the ZR viral RNA kit
471 (ZymoResearch). The sequences of the primers used in qPCR are shown in
472 **Supplementary Table 2.**

473

474 **Humanized Mice**

475 NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/Jic (NOD/SCID *Il2rg*^{null}) mice were obtained from the
476 Central Institute for Experimental Animals (Kanagawa, Japan). The mice were
477 maintained under specific-pathogen-free conditions and were handled in accordance with
478 Regulations on Animal Experimentation at Kyoto University. The study protocol was
479 approved by the Animal Experimentation Committee in Kyoto University. Human
480 CD34⁺ hematopoietic stem cells (HSCs) were isolated from human fetal liver as
481 previously described⁴⁹. To generate humanized mice (NOG-hCD34 mice), human fetal
482 liver-derived CD34⁺ cells (5 \times 10⁴ to 12 \times 10⁴ cells) were intrahepatically injected into
483 newborn NOG mice aged 0 to 2 days after X-irradiation (10 cGy per mouse) in an
484 RX-650 X-ray cabinet system (Faxitron X-ray Corporation). Humanized mice were
485 randomly assigned to HIV-1 infection or mock treatment.

486

487 **Generation of N4BP1 or MALT1 Knockout Cells by the CRISPR/Cas9 System**

488 N4BP1 knockout Jurkat cells were generated by transiently transfecting

489 pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene) with Neon Transfection
490 System according to the manufacturer's protocol. N4BP1 coding exon was targeted
491 using the following sgRNA target site: 5'- AGATATAAAAGAACTACTG -3'. Single
492 clones were obtained by limiting dilution in 96-well U-bottomed culture plates. Control
493 cells were obtained following transfection of the plasmid without sgRNA. For
494 generating MALT1 knockout Jurkat cells, cells were delivered with the sgRNA and
495 Cas9 expressing lentivirus vector (LentiCrispr v2 puro: Addgene) targeting the MALT1
496 coding sequences; sgRNA1 5'-GCAGTGCATGTAAAAGATGC-3', sgRNA2
497 5'-ATTCAGCCAGTGGTCACAGC-3'. Control cells were obtained by transduction of
498 lenticrispr v2 expressing non-targeting control sgRNA; 5'-
499 GGCCGATAATGATCCGACCG -3. Two days after transduction, cells were cultured
500 with 1µg/ml Puromycin for 10 days. Knockout of N4BP1 or MALT1 was examined by
501 immunoblot analysis.

502

503 **Establishment of Stably N4BP1 Expressing Cells**

504 Stably N4BP1 expressing Jurkat cells were generated by transfection with CSII Bsd
505 IRES MCS-N4BP1 using the Neon Transfection System. Three days after transfection,
506 N4BP1 expressing cells were selected by culturing them in 10 µg/ml
507 blasticidin-containing RPMI-1640 for 14 days. Control cells were prepared by
508 transfecting an empty plasmid. In some experiments, single clones were obtained by
509 limiting dilution in 96-well U-bottomed culture plates. Stably N4BP1 expressing
510 HEK293T cells and control cells were generated by transfection with CSII Bsd IRES
511 MCS-N4BP1 and empty plasmid, respectively. For inducible expression of N4BP1,
512 N4BP1 KO cells, JNLGFP, N4BP1 KO JNLGFP or J-Lat10.6 cells were prepared by
513 pseudotyped lentivirus vector transduction with pInducer20 vector with or without
514 wild-type or R509A mutant N4BP1 followed by selection in G418.

515

*D. Yamasoba et al.***516 siRNA Transfection and Infection of Macrophages and Jurkat cells**

517 On days 7 and 10 of differentiation, MDM were transfected with N4BP1-specific or
518 non-targeting control siRNA using Lipofectamine RNAiMAX (Life technologies)
519 followed by infection at day 10. All siRNAs were provided in lyophilized state by
520 ThermoFisher (#18638, #18639, #18640) or Eurofins Genomics (non-targeting control:
521 UUCUCCGAACGUGUCCACGdTdT) and suspended in nuclease-free water to reach
522 a final concentration of 20 μ M. siRNA transfection was performed in 12-well plates with
523 three technical replicates for each sample. For one well, 2.25 μ l siRNA were mixed with
524 150 μ l Opti-MEM and 6 μ l Lipofectamine RNAiMAX were mixed with 150 μ l
525 Opti-MEM. These two solutions were then mixed and incubated at room temperature for
526 10 min. Afterwards, 300 μ l of the mixture was dropped on the well containing 1 ml of cell
527 culture medium. Medium was changed 18 h after each transfection. 3 and 6 days post
528 infection, macrophage culture supernatants were harvested and used to infect TZM-bl
529 reporter cells. To this end, 6,000 TZM-bl cells were sown in 96-well plates and infected
530 in triplicate with cell culture supernatants containing infectious virus. Three days later,
531 infection rates were determined using a galactosidase screen kit (GalScreen-Applied
532 Bioscience) according to the manufacturer's instructions. β -galactosidase activities were
533 quantified as relative light units per second (RLU/s) using an Orion Microplate
534 Luminometer. For knockdown in Jurkat cells, cells were transfected with N4BP1-specific
535 siRNA (s18640) or negative control siRNA using NEON according to the manufacturer's
536 protocol. Differentiated human macrophages were transfected with siRNA on days 6 and
537 8 after isolation. Cells were transfected with N4BP1-specific siRNA (s18638-18640) or
538 negative control siRNA using Lipofectamin RNAiMAX (Life technologies) according to
539 the manufacturer's instructions.

540

541 Structure Modeling.

542 The PIN RNase domain of human N4BP1 was modeled with the SFAS threading meta

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server (<http://sysimm.ifrec.osaka-u.ac.jp/sfas2/>) using human Regnase-1 (PDB Identifier 3v32, chain B) as a template. The conservation heatmap was constructed by aligning the top 1000 hits from the NCBI nr database to human N4BP1 using MAFFT (PMID: 23329690), and computing the sequence identity to human N4BP1. The sequence identity was expressed as a temperature factor in the human N4BP1 model PDB file and displayed in PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.).

549 **Generation of Recombinant N4BP1 and N4BP1-D623N Proteins**

HEK293T cells were transfected with pEFs_Flag-SBP-N4BP1 or pEFs_Flag-SBP-N4BP1-D623N using polyethyleneimine “MAX” (Polysciences). Three days after transfection, cells were lysed in NF-lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 0.25 M Sucrose, 0.5% (v/v) NP-40, 1% (v/v) Tween 20] containing 1 mM DTT, protease inhibitor cocktail (Nacalai Tesque), phosphatase inhibitor cocktail (EDTA free) (Nacalai Tesque) and 50 µg/ml RNase A. SBP-tagged proteins were captured by Streptavidin Mag Sepharose (GE Healthcare) and eluted with T buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM MgCl₂, 0.05% (v/v) Tween 20) containing 1 mM DTT, 0.1 x protease inhibitor cocktail (EDTA free) (Nacalai Tesque), 0.1 x phosphatase inhibitor cocktail (Nacalai Tesque) and 2 mM d-desthiobiotin (Sigma-Aldrich).

562 ***In vitro* RNA Cleavage Assay**

The *in vitro* RNA cleavage assay has been previously described³⁹. Briefly, recombinant N4BP1 protein and *in vitro* transcribed 5'-[³²P]-labelled viral RNA (*tat*, *rev* and *nef* subgenomic RNA of HIV-1 NL4-3)⁵⁰ or a 513 base RNA fragment from the pBluescript® vector) were mixed in cleavage buffer (25 mM HEPES, 50 mM potassium acetate, 5 mM DTT, 5 mM magnesium acetate and 0.2 U/ml RNasin (Promega)) for 60 min at 37 degrees. The cleaved RNA was analyzed by denaturing 6% polyacrylamide-TBE-urea gels (Invitrogen) and autoradiography. The sequence used for

570 *in vitro* transcription and the *in vitro* cleavage assay is shown in **Supplementary Table 3.**

571

572 **Northern Blotting.**

573 Using Trizol reagent, total RNA was isolated from HEK293T cells 48 hours
574 post-transfection with pNL4-3 together with N4BP1 expression plasmid, D623N or
575 empty plasmid. Extracted RNA was electrophoretically separated, transferred to
576 Hybond-N+ (GE healthcare), and hybridized with the probe derived from a fragment of
577 pNL4-3. ³²P-labeled probe was generated from the 422-nt XhoI/BamHI restriction
578 fragment in the 3' UTR of pNL4-3, which is present in all HIV-1 mRNAs. HIV-1 primary
579 RNA transcripts, generated by alternative splicing, were detected as three major bands
580 representing three different sizes.

581

582 **RNA-Immunoprecipitation and qPCR Analysis**

583 HEK293T cells seeded at 3×10^6 in 10 cm plates were transfected with 5 µg of pNL4-3
584 together with 5 µg of a plasmid expressing the N4BP1 D623N mutant. Flag-tagged
585 N4BP1 was immunoprecipitated with an anti-Flag antibody (Sigma) or control mouse
586 IgG isotype controls (Thermo Fisher) 48 hours after transfection. N4BP1 interacting
587 HIV-1 RNA was extracted using Trizol, quantified by RT-qPCR and normalized to 18S
588 RNA bound in a non-specific manner.

589

590 **Analysis of Global Protein Synthesis**

591 Control and N4BP1 KO Jurkat cells were cultured for 1 day. As negative control, some
592 control cells were treated with a protein synthesis inhibitor, Cycloheximide (Cayman
593 Chemical) for 30 min. Then the cells were harvested, and translating polypeptides were
594 labeled with O-Propargyl-Puromycin (OPP) for 30 min at 37 °C followed by staining
595 with 5 FAM Azide using the Protein Synthesis Assay kit (Cayman Chemical) according
596 to the manufacturer's instruction. The cells were analyzed by Flow cytometry

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597 (FACSVerse; BD). The data analysis was performed using FlowJo (LCC).

598

599 **HIV-LTR Reporter Gene Assay.**

600 TZM-bl cells were transfected with pGL3-HIV-LTR-Luc plasmid or pGL3-empty
601 plasmid together with N4BP1 expression plasmid or empty control plasmid. 24 h
602 post-transfection, cells were lysed and luciferase activities in the lysates were determined
603 using the Dual-luciferase reporter assay system (Promega).

604

605 **Detection of Early RT products, Late RT product and Integrated Proviral DNA**

606 Quantification of HIV-1 early RT (R/U5), late RT (U5/gag) and integrated products by
607 real-time PCR was done by following a published protocol (Suzuki et al., 2003). Briefly,
608 virus was treated with DNase-I (TAKARA) at a concentration of 20 mg/ml in the
609 presence of 10mM MgCl₂ at the room temperature. Heat-inactivated (65°C, 30 min)
610 virus was used as a negative control for infection. Jurkat cells were transfected with
611 N4BP1 or control siRNA. One day after transfection, Jurkat cells were exposed to
612 HIV-1 NL4-3 (MOI 0.1) or heat inactivated HIV-1 at 37°C for 2 hours. Total DNA was
613 isolated 12 hours after infection, by using DNeasy Blood & Tissue Kits (QIAGEN)
614 according to the manufacturer's instructions. Early RT products, late RT products and
615 integrated DNA were quantified by real-time PCR as described previously (Suzuki et al.,
616 2003).

617

618 **Influenza Infection Experiment**

619 HEK293T cells stably expressing N4BP1 or control plasmid were infected with
620 Influenza A virus PR8 or WSN strain for 24 hours before total RNA or culture media
621 were harvested. Total RNA was subjected to qPCR analysis to measure the expression
622 levels of viral mRNA for segment 4 (HA). The viral growth in the culture media was
623 titrated by using plaque assays on Madin–Darby canine kidney (MDCK) cells.

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624

625 **Statistical Analysis and Reproducibility.**

626 Statistical analyses were conducted using Prism 8 (GraphPad, La Jolla, CA, USA) or
627 Excel for Office365. Statistical significance was calculated with an unpaired two-tailed
628 Student's *t*-test. Data are presented as the mean \pm s.d.. A *P* value of < 0.05 was considered
629 statistically significant. The screening of RBPs restricting HIV-1 was repeated and the
630 results were confirmed in an independent experiment (Fig. 1a). The *in vivo* experiment
631 with humanized mice (Fig. 2e) was performed once. The data for HIV-1 yield in MDM
632 treated with N4BP1-specific siRNAs (Fig. 3g) and p24 production comparing between
633 WT or R509A mutant N4BP1 reconstituted JNLGFP cells (Fig. 6h) are pooled from
634 three independent experiments. Other *In vitro* experiments were representative of 2-5
635 independent experiments with similar results.

636

637 **Data Availability**

638 The data that support the findings of this study are available from the corresponding
639 author upon request.

640

641

642

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664

665 **Author Contributions**

666 D.Y., K.S., D.S. and O.T. designed the study. D.Y. designed, carried out and analyzed
667 experiments. T.I., T.I., L.K., S.J., E.R., D.H., N.M, K.A, T.U and T.M. provided
668 technical and intellectual assistance. S.M. and T.N. conducted influenza infection
669 experiments. A.Y. provided recombinant proteins. D.S. performed structural modeling

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670 and bioinformatic analysis. D.Y., D.S. and O.T. wrote the manuscript. K.S, F.K., Y.K.

671 and O.T. supervised the study.

672

673 **Competing interests**

674 The authors declare no competing financial interests.

675

676

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- 816
- 817

818 **Figure Legends**

819 **Fig. 1. Identification of N4BP1 as an antiretroviral restriction factor.**

820 **a**, HEK293T cells were co-transfected with pNL4-3 and one of 62 expression plasmids
821 (Supplementary Table 1) encoding putative RNA binding proteins. Forty-eight hours post
822 transfection, cell culture supernatants were harvested and used to infect TZM-bl reporter
823 cells to determine infectious virus yield. The data values of two technical replicates.

824 **b**, HEK293T cells were transfected with either pNL4-3 or pAD17 together with
825 increasing amounts of N4BP1 expression plasmid. Expression of HIV-1 Env and Gag,
826 and N4BP1 in cell lysates as well as Gag p24 in the culture supernatants was determined
827 by immunoblot analysis 48 hours post-transfection. β -Actin was used as the loading
828 control. The upper panel shows infectious virus yield relative to the empty vector control
829 determined by the TZM-bl reporter assay. $n = 3$ biological replicates. Individual points
830 and means \pm s.d. are shown.

831 **c**, HEK293T cells were co-transfected with proviral clones of HIV-1 together with
832 increasing amounts of vector expressing N4BP1. 48 hours post-transfection, a TZM-bl
833 reporter assay was performed to measure the production of infectious virus in
834 supernatants. Infectious virus yield relative to the empty vector control is shown as mean
835 \pm s.d of biological replicates ($n = 3$). TF, transmitted founder virus; CC chronic control
836 virus; 6-mo, virus isolated 6 months post infection.

837 P values were calculated using unpaired two-tailed Student's t -test. * $P < 0.05$; *** $P <$
838 0.005.

839

840 **Fig. 2. N4BP1 is upregulated upon IFN stimulation and restricts viral replication in**
841 **T cells.**

842 **a**, *N4BP1* mRNA levels were measured by RT-qPCR in Jurkat cells treated with IFN- α
843 from human leukocytes (1000 U/ml) for the indicated periods of time. Data are shown as
844 mean \pm s.d. of biological replicates ($n = 3$).

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845 **b**, Immunoblot analysis of N4BP1 in cell lysates from Jurkat cells treated with IFN- α
846 for 48 hours. β -Actin was used as the loading control.

847 **c**, *N4BP1* mRNA levels were measured by RT-qPCR in primary human CD4⁺ T cells
848 treated with IFN- α for 24 h. Individual points and means \pm s.d. are shown (n = 3). *P <
849 0.05; ***P < 0.005.

850 **d**, Expression levels of *IFNBI*, *N4BP1*, *BST-2* and *ISG15* mRNAs were measured by
851 qPCR in Jurkat cells infected with HIV-1 NL4-3 (MOI 0.01). Data are shown as mean \pm
852 s.d. of biological replicates (n = 3).

853 **e**, *N4BP1* mRNA levels in spleens from humanized mice 6 weeks after HIV-1 infection (n
854 = 8) or mock treatment (n = 6).

855 **f**, Immunoblot analysis of N4BP1 in cell lysates from control and N4BP1-knockout
856 Jurkat cells (left panel). N.S. Non-specific. Replication of HIV-1 NL4-3 (MOI 0.01) in
857 N4BP1 knockout or control Jurkat cell lines (right panel). Infectivity of HIV-1 in the
858 culture supernatants was measured by TZM-bl assay.

859 **g**, Immunoblot analysis of N4BP1 in cell lysates from control cells and N4BP1-knockout
860 Jurkat cells inducibly reconstituted with N4BP1 by using the Tet-on system and
861 doxycycline stimulation (Dox) for 24 hours (left panel). Replication of HIV-1 NL4-3
862 (MOI 0.01) in control or N4BP1 knockout Jurkat cell lines reconstituted with or without
863 N4BP1 by Dox treatment (right panel). Infectivity of HIV-1 in the culture supernatants
864 was measured by TZM-bl assay.

865 **h**, Immunoblot analysis of Jurkat cells stably expressing FLAG-tagged N4BP1 (left
866 panel). Replication of HIV-1 NL4-3 (MOI 0.01 or 0.001) in FLAG-N4BP1 expressing or
867 control Jurkat cell lines (right panel). Infectivity of HIV-1 in the culture supernatants was
868 measured by TZM-bl assay.

869 *P* values were calculated using unpaired two-tailed Student's *t*-test. *P < 0.05; **P < 0.01;
870 ***P < 0.005.

871

Fig. 3. N4BP1 is upregulated upon IFN stimulation and restricts HIV-1 infection in macrophages

a, Expression levels of *N4BP1* or *BST-2* mRNA were measured by RT-qPCR in macrophage-like THP-1 cells stimulated with IFN- α for the indicated periods of time. Data are shown as mean \pm s.d. of biological replicates ($n = 3$).

b, Immunoblot analysis of N4BP1 and β -Actin in cell lysates from macrophage-like THP-1 cells stimulated with IFN- α for 48 hours.

c, Expression levels of *N4BP1* mRNA were measured by RT-qPCR in human primary MDMs stimulated with IFN- α for 24 hours. $n = 3$ biological replicates. Individual points and means \pm s.d. are shown.

d, Expression levels of N4BP1 in human primary MDMs upon stimulation of IFN- α for 48 hours were determined by immunoblotting.

e, N4BP1 expression levels were normalized to β -Actin levels and the unstimulated sample was set to 100 %. $n = 3$ biological replicates. Individual points and means \pm s.d. are shown.

f, Representative immunoblot analysis of human MDMs transfected with control siRNA or three different N4BP1-specific siRNAs (left panel). N4BP1 expression levels were normalized to β -Actin levels and the control siRNA sample was set to 100 %. Data are shown as mean values \pm s.d. of 5 independent experiments.

g, Human MDMs treated with control or N4BP1-specific siRNA (#18638-#18640) were infected with HIV-1 AD8 and analyzed for infectious virus production 3 ($n = 9$) and 6 ($n = 7$) days post-infection (dpi). Shown are mean percentages \pm s.d. relative to those detected in control cells (100%).

P values were calculated using unpaired two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Fig. 4. N4BP1 is a cellular RNase degrading HIV-1 RNA

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- 899 **a**, The expression levels of viral mRNA were measured by RT-qPCR in HEK293T cells
900 cotransfected with an N4BP1 expression plasmid or an empty vector control together
901 with the indicated viral infectious clones. n = 3 biological replicates. Individual points
902 and means \pm s.d. are shown.
- 903 **b**, Domain architecture of human N4BP1.
- 904 **c**, A structural modeling of the RNase domain of N4BP1. Colors highlight the
905 evolutionary conservation of amino acids. D623, D704, D705 and D723 forming the
906 catalytic center of the RNase are indicated (Left panel). Amino acid sequence alignment
907 of the partial RNase domain of N4BP1 orthologs from various species. “*”Fully
908 conserved residue, “:”conservation between groups of strongly similar properties, “.”
909 conservation between groups of weakly similar properties (right panel).
- 910 **d**, Purified N4BP1, but not its D623N mutant, cleaves [32 P]-labeled RNA derived from a
911 subgenomic sequence of HIV-1 NL4-3 *in vitro*.
- 912 **e**, Structural models of the RNase domains (residues 616-775) in WT and D623N human
913 N4BP1.
- 914 **f**, Northern blot analysis of HIV viral RNAs in HEK293T cells co-transfected with
915 pNL4-3 and vectors expressing N4BP1 or N4BP1 D623N. Ribosomal 28S and 18S
916 RNAs were included as loading controls.
- 917 **g**, RNA-IP-qPCR assay in HEK293T cells transfected with pNL4-3 together with or
918 without Flag-tagged N4BP1. Flag-tagged N4BP1 D623N was immunoprecipitated with
919 anti-Flab Ab or control IgG 48 hours after transfection and co-precipitated RNAs were
920 quantified by RT-qPCR. n = 3 biological replicates. Individual points and means \pm s.d. are
921 shown.
- 922 **h**, HEK293T cells were cotransfected with pNL4-3 and vectors expressing N4BP1 or
923 N4BP1 D623N. Shown are the immunoblots of cellular extracts and viral particles in the
924 culture supernatants.
- 925 **i**, Infectious virus yield of HIV-1 NL4-3 in HEK293T cells transfected with pNL4-3

926 together with expression plasmids for N4BP1 WT, N4BP1 D623N or an empty vector
927 control as assessed by TZM-bl reporter assay. Infectious virus yield relative to the empty
928 vector control is shown. $n = 3$ biological replicates. Individual points and means \pm s.d. are
929 shown.
930 P values were calculated using unpaired two-tailed Student's t -test. * $P < 0.05$, ** $P <$
931 0.001 , *** $P < 0.005$.

932

933 **Fig. 5. TCR stimulation induces MALT1-mediated degradation of N4BP1**

934 **a**, Immunoblot analysis of N4BP1 levels in primary $CD4^+$ T cells treated with
935 IFN- γ (50 ng/ml), IL-2 (100 U/ml), PMA (50 ng/ml) plus ionomycin (500 nM) or
936 anti-CD3/CD28 antibody-coated beads for 24 hours.

937 **b**, N4BP1 protein levels in Jurkat cells stimulated with PMA plus ionomycin for the
938 indicated periods of time were determined by immunoblotting.

939 **c**, Immunoblot analysis of N4BP1 in parental or MALT1-deficient Jurkat cells stimulated
940 with PMA plus ionomycin for 1 hour.

941 **d**, N4BP1 protein levels in Jurkat cells stimulated with PMA plus ionomycin for 1 hour
942 with zVAD-fmk, a pan-caspase inhibitor (10 μ M) or zVRPR-fmk, a MALT1 inhibitor
943 (100 μ M).

944 **e**, Schematic representations of N4BP1 and its mutants. The epitope of the
945 N4BP1-specific antibody used in the present study is indicated.

946 **f**, The apparent molecular weight of N4BP1 and its truncation mutants was determined by
947 immunoblotting of transfected HEK293T cells.

948 **g**, Sequence-logo plot representing the amino acid frequencies at positions P4 to P3' in 16
949 previously identified MALT1 cleavage sites (Supplementary Fig. 10e).

950 **h**, Localization of the potential MALT1 cleavage site in N4BP1. Multiple sequence
951 alignment of different N4BP1 orthologs. “*” Fully conserved residue, “.” conservation
952 between groups of weakly similar properties.

953 **i**, Cleavage of N4BP1 in HEK293T cells transfected with the indicated N-terminally
954 Flag-tagged N4BP1 variants. Degradation of N4BP1 and emergence of the 72 kDa
955 cleavage product were monitored by immunoblot with an anti-Flag antibody.

956 **j**, N4BP1 levels were determined by immunoblotting of Jurkat cells transfected with an
957 empty vector or expression plasmids for Flag-N4BP1.

958 **k**, HEK293T cells were co-transfected with pNL4-3 and vectors expressing WT or
959 mutant N4BP1. 48 hours post-transfection, a TZM-bl reporter assay was performed.
960 Infectious virus yield relative to the empty vector control is shown. $n = 3$ biological
961 replicates. Individual points and means \pm s.d. are shown.

962 P values were calculated using unpaired two-tailed Student's t -test. *** $P < 0.005$.

963

964 **Fig. 6. MALT1-mediated degradation of N4BP1 in latently HIV-1 infected cells**
965 **contributes to viral reactivation.**

966 **a-b**, Immunoblot analysis of N4BP1 and MALT1 in cell lysates from MALT1-deficient
967 J-Lat10.6 (**a**), JNLGFP (**b**) or control cells stimulated with or without PMA (50 ng/ml) for
968 24 hours.

969 **c-d**, The expression levels of *tat/rev* and *gag* mRNA were quantified by qPCR in
970 MALT1-deficient J-Lat10.6 (**c**) or JNLGFP cells (**d**) stimulated with PMA (50 ng/ml) for
971 the indicated periods of time. Data are shown as mean \pm s.d. of biological replicates ($n =$
972 3).

973 **e-f**, Immunoblot analysis of N4BP1 and Gag p24 proteins in cell lysates or culture
974 supernatants from J-Lat10.6 (**e**) or JNLGFP cells (**f**), which inducibly express N4BP1 or
975 N4BP1 R509A by the Tet-on system treating with Dox and were stimulated with PMA
976 (10 ng/ml) for 24 hours. The levels of Gag p24 in the supernatants were quantified and
977 indicated as the percentage of Dox (-) controls.

978 **g**, Immunoblot analysis of N4BP1 in control and N4BP1 KO JNLGFP cells generated
979 by the CRISPR/Cas9 system. β -Actin was used as loading control.

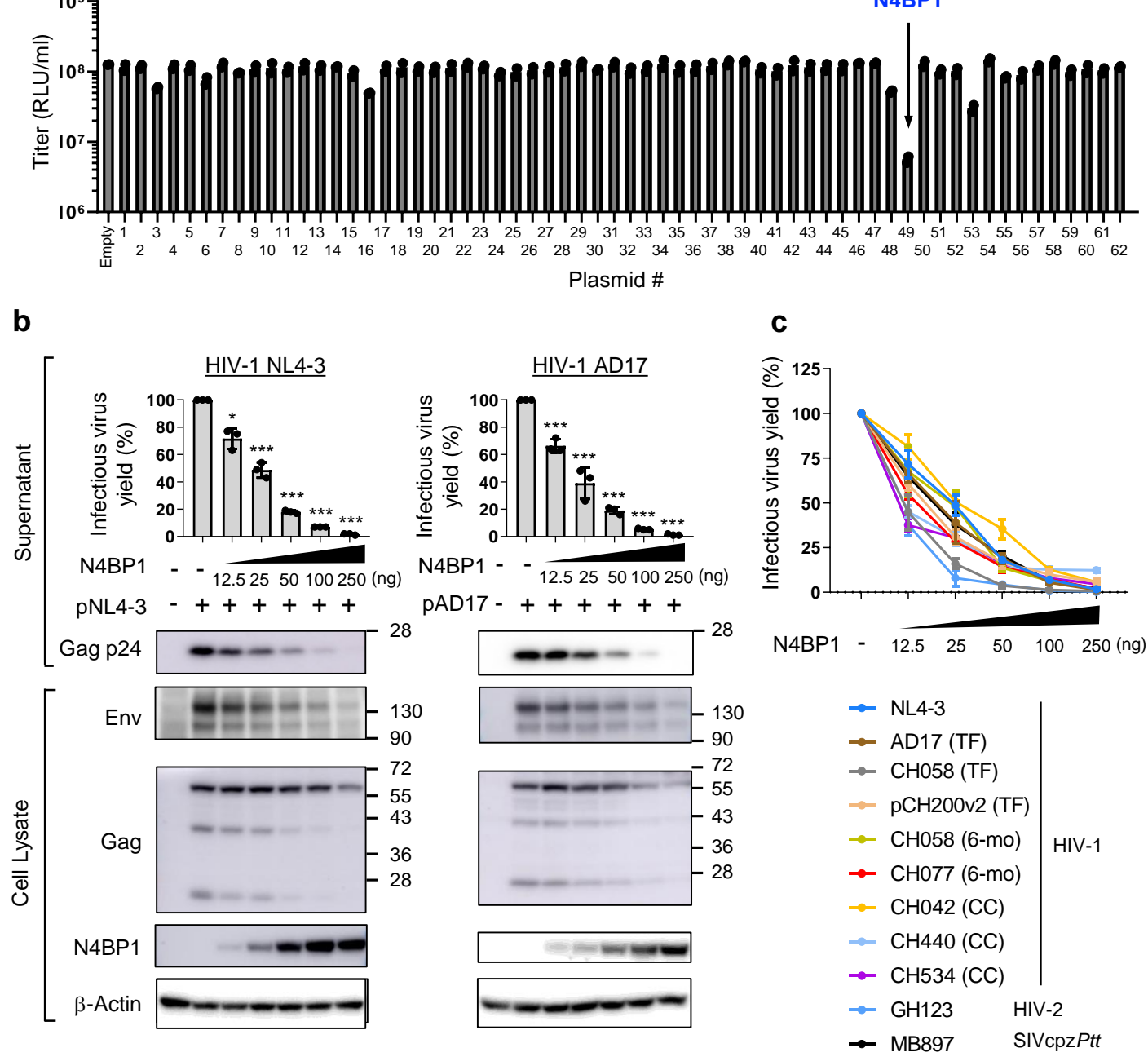
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980 **h-i**, N4BP1 KO JNLGFP cells were inducibly reconstituted with WT or R509A N4BP1
981 by the Tet-on system via Dox treatment, followed by stimulation with PMA (10 ng/ml)
982 for 24 hours. Cell lysates and culture supernatants were collected and immunoblot
983 analysis was performed to determine the expression of N4BP1, Gag and β -Actin
984 proteins (**h**). The ratio of p24 expression in the culture supernatants between Dox (-)
985 controls and Dox (+) WT or R509A N4BP1 expressing cells examined in (**h**) is shown
986 in (**i**) ($n = 3$). Individual points and means \pm s.d. are shown.
987 P values were calculated using unpaired two-tailed Student's t -test. * $P < 0.05$, ** $P <$
988 0.001, *** $P < 0.005$.

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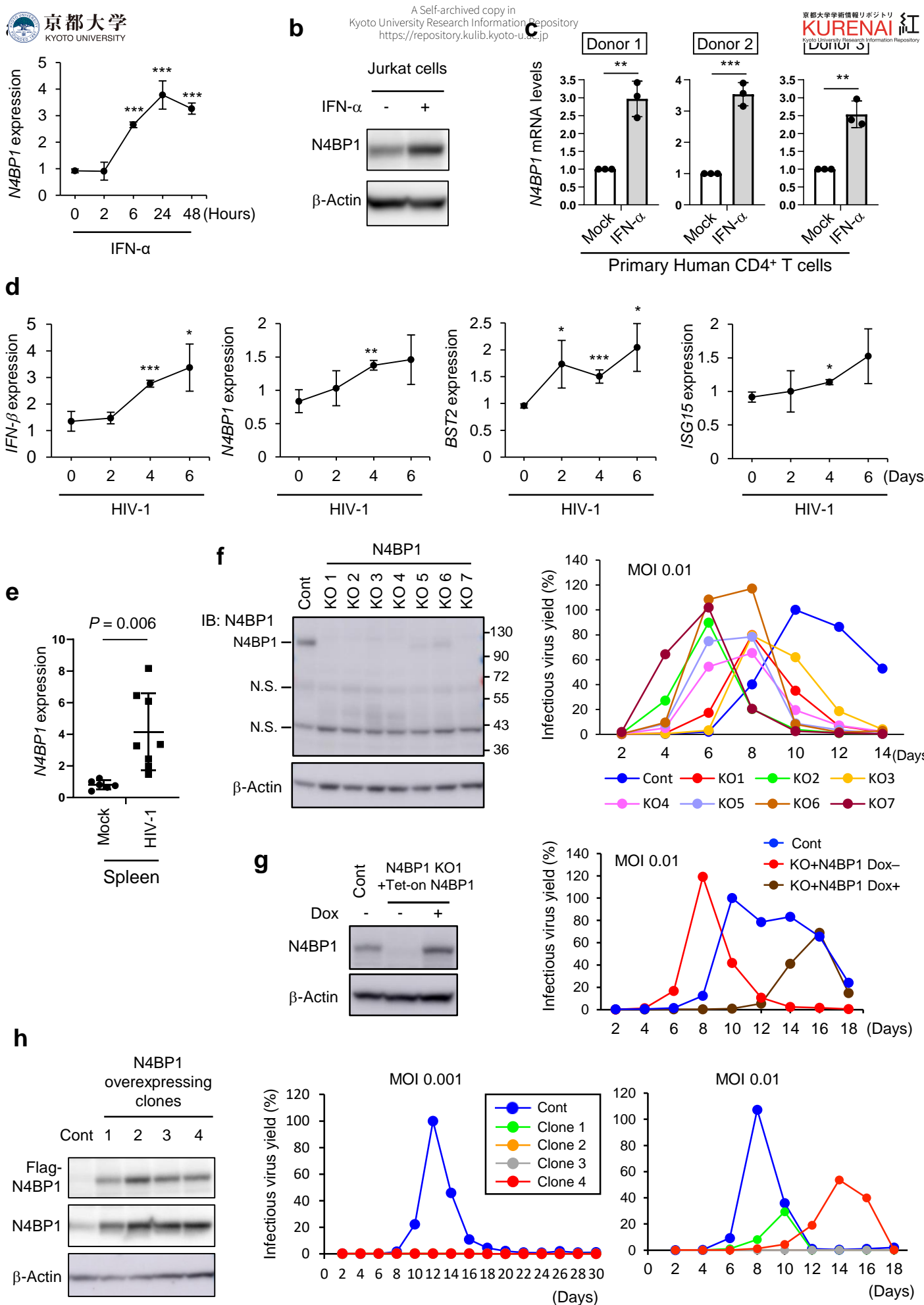


Figure2

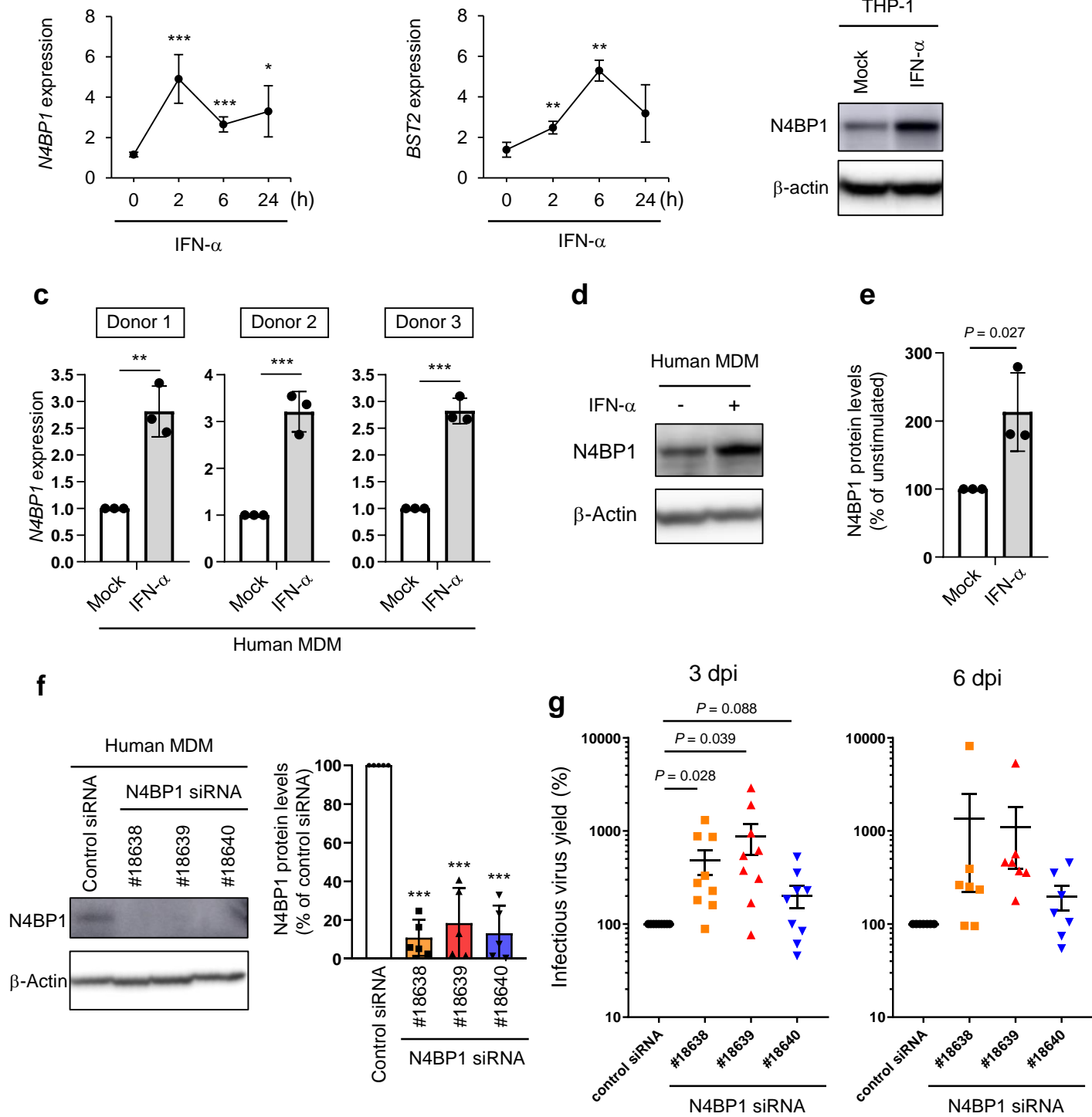


Figure 3



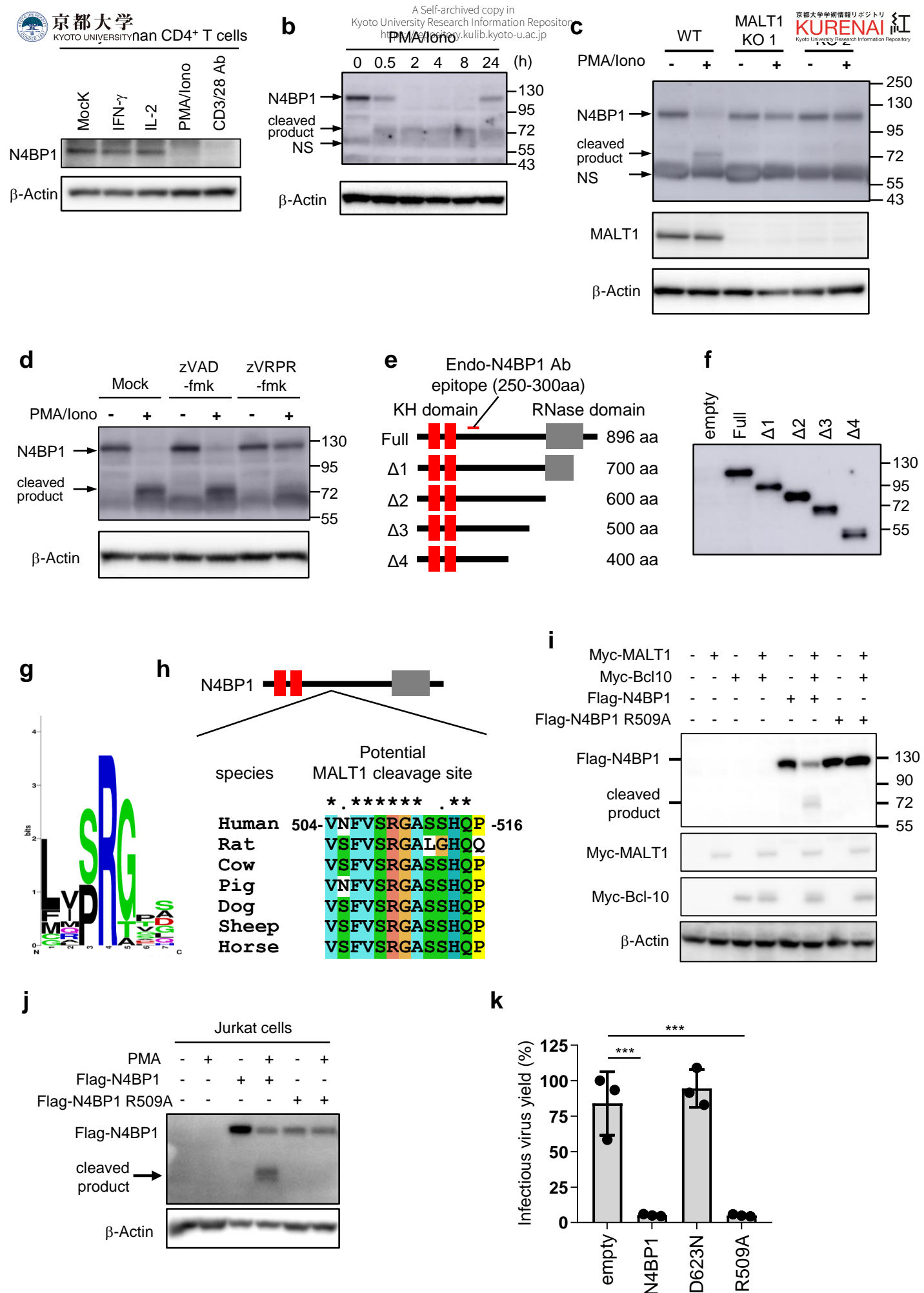


Figure 5



Supplemental Information for

N4BP1 restricts HIV-1 and its inactivation by MALT1 promotes viral reactivation

Daichi Yamasoba^{1,2,5}, Kei Sato^{3,6,7}, Takuya Ichinose^{1,2,5}, Tomoko Imamura², Lennart Koepke⁸, Simone Joas⁸, Elisabeth Reith⁸, Dominik Hotter⁸, Naoko Misawa³, Kotaro Akaki^{1,2}, Takuya Uehata^{1,2}, Takashi Mino^{1,2}, Sho Miyamoto⁴, Takeshi Noda⁴, Akio Yamashita⁹, Daron M. Standley¹⁰, Frank Kirchhoff⁸, Daniel Sauter⁸, Yoshio Koyanagi³ and Osamu Takeuchi^{1,2*}

¹Department of Medical Chemistry, Graduate School of Medicine,
²Laboratory of Infection and Prevention, ³Laboratory of Systems Virology, ⁴Laboratory of Ultrastructural Virology, Institute for Frontier Life and Medical Sciences, ⁵Graduate School of Biostudies, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

⁶CREST, Japan Science and Technology Agency, Saitama 322-0012, Japan.

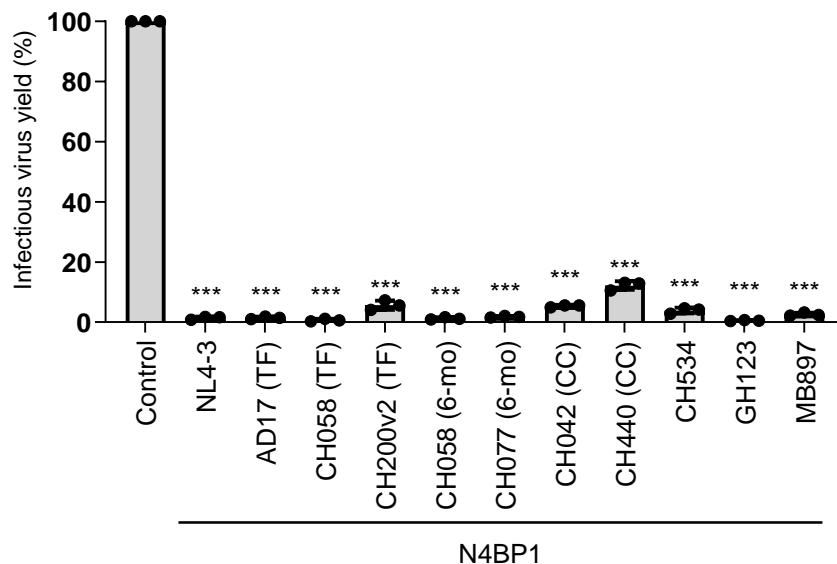
⁷Department of Systems Virology, Institute for Medical Science, the University of Tokyo, 4-6- 1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

⁸Institute of Molecular Virology, Ulm University Medical Center, 89081 Ulm, Germany

⁹Department of Molecular Biology, Yokohama City University School of Medicine, Kanagawa 236-0004, Japan

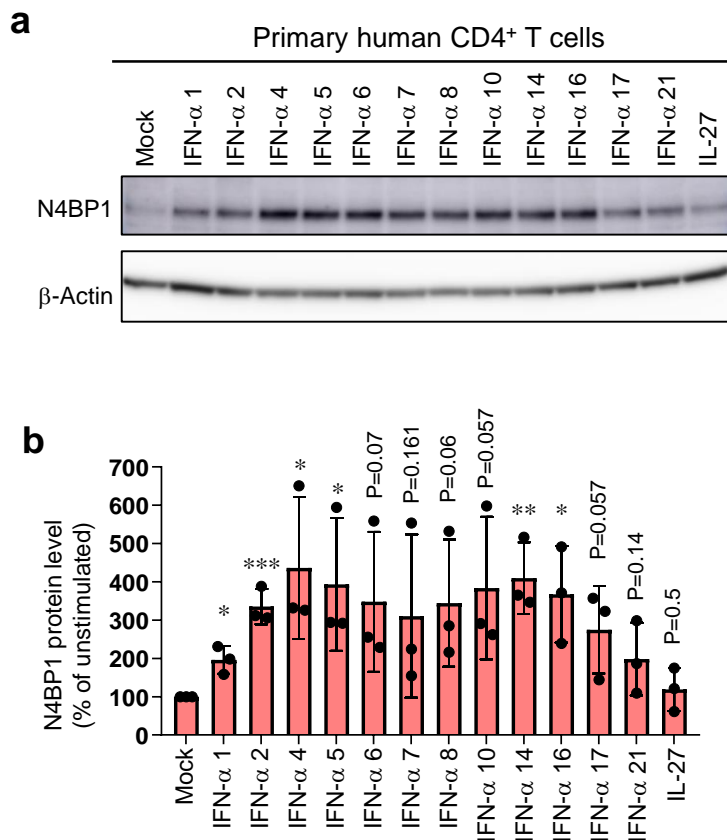
¹⁰Department of Genome Informatics, Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, 3 Yamada-oka, Suita, Osaka 565-0871, Japan

*e-mail: otake@mfour.med.kyoto-u.ac.jp.



Supplementary Figure 1. N4BP1 inhibits various clones of HIV-1, HIV-2 and SIVcpzPtt.

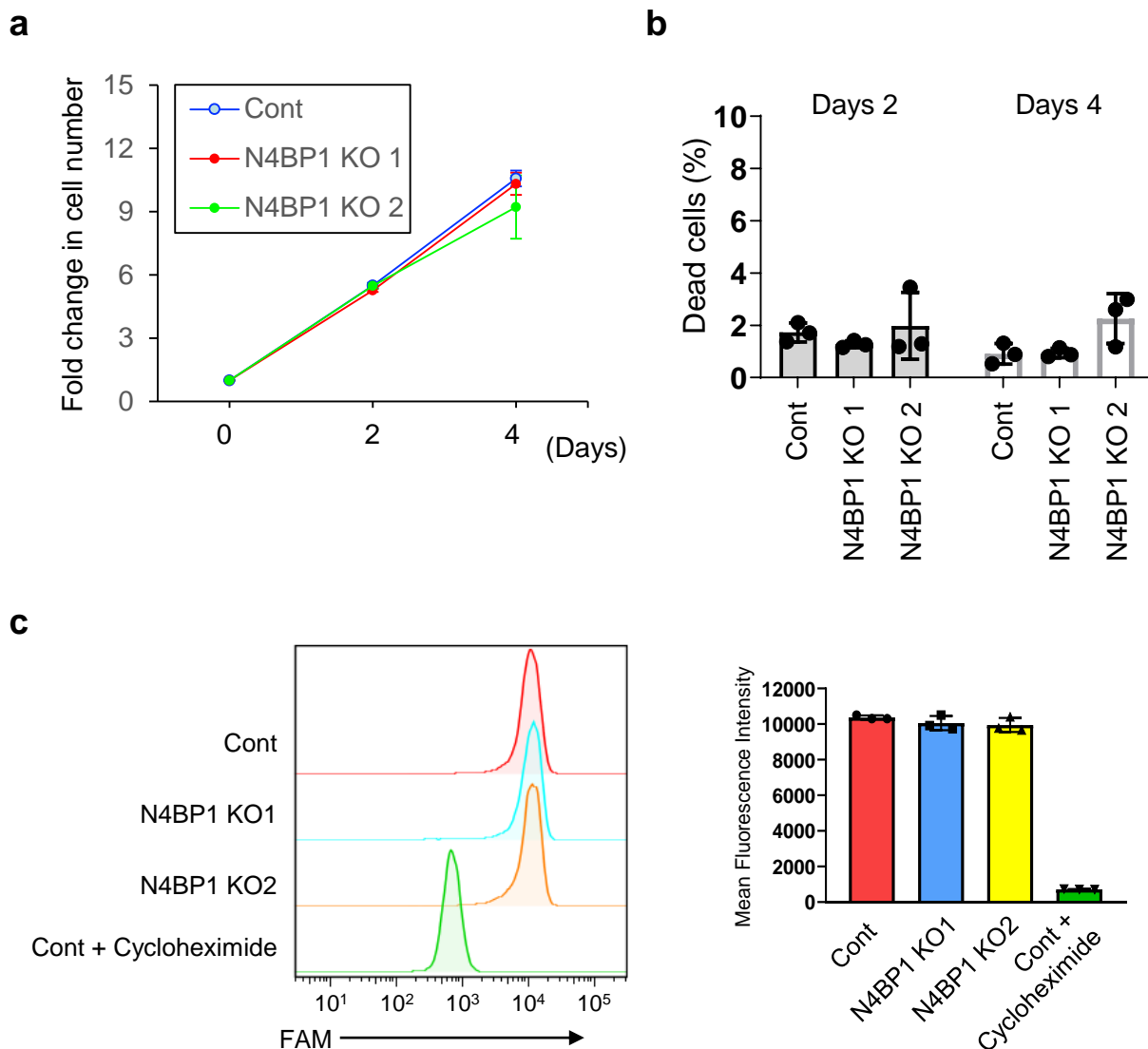
HEK293T cells were transfected with proviral clones expressing NL4-3, AD17 (TF), CH058 (TF), CH200v2 (TF), CH058 (6-mo), CH077 (6-mo), CH042 (CC), CH534 (CC), CH440 (CC), GH123 (HIV-2) or MB897 (SIVcpzPtt) (250 ng each), together with the N4BP1 expression plasmid (250 ng). Forty eight hours post-transfection, a TZM-bl reporter assay was performed to measure the production of infectious virus in the cell culture supernatants. Infectious virus yields normalized to the empty vector control are shown as mean values \pm s.d. of biological replicates ($n = 3$). P values were calculated using unpaired two-tailed Student's t -test. *** $P < 0.005$.



Supplementary Figure 2. N4BP1 expression is induced by various IFN- α subtypes in human primary CD4⁺ T cells.

(a) Immunoblot analysis for the expression of N4BP1 in human primary CD4⁺ T cells stimulated with indicated IFN- α subtypes (50 ng/ml each) or IL-27 (5 ng/ml) for 3 days. β -Actin was used as loading controls. Data are representative of three independent experiments.

(b) N4BP1 expression levels were determined by immunoblotting as shown in (a) and normalized to β -actin levels. The unstimulated sample was set to 100%. Data shown are mean values \pm s.d. of immunoblot data derived from 3 individual donors. P values were calculated using unpaired two-tailed Student's t -test. * P < 0.05; ** P < 0.01; *** P < 0.005.

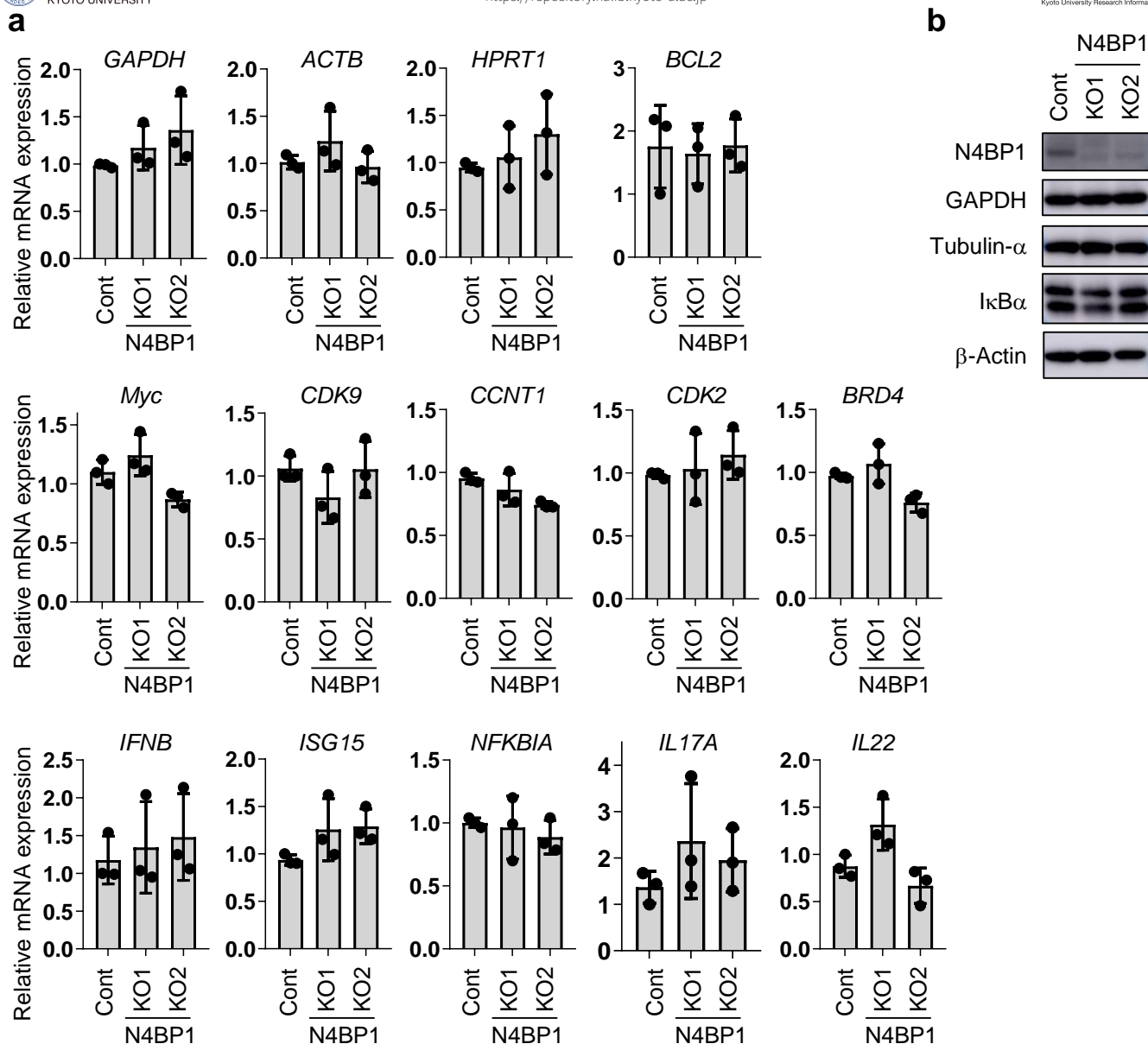


Supplementary Figure 3. N4BP1 deficiency in Jurkat cells does not affect growth, cell death or global protein synthesis

(a) N4BP1 KO Jurkat cells (clones 1 and 2) and control (Cont) cells were cultivated for 2 and 4 days and changes in cell numbers are shown. Data are shown as mean \pm s.d. of biological replicates ($n = 3$).

(b) Cells in a were analyzed by Flow cytometry (FACSVerse; BD) after propidium iodide staining. Individual points and means \pm s.d. are shown ($n = 3$).

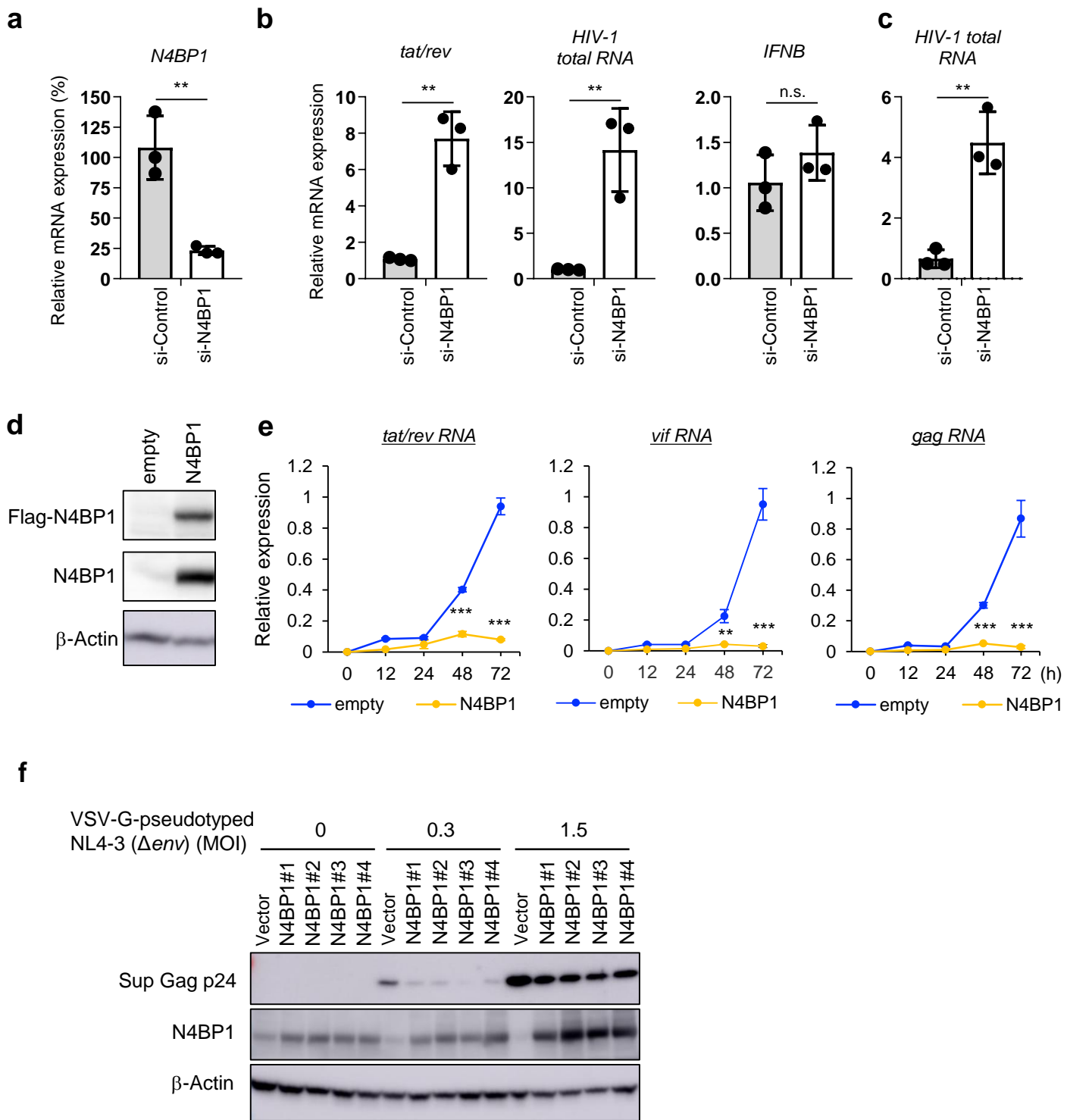
(c) Control and N4BP1 KO Jurkat cells were cultured for 1 day. As negative control, some control cells were treated with a protein synthesis inhibitor, Cycloheximide (Cayman Chemical) for 30 min. Then the cells were harvested, and translating polypeptides were labeled with O-Propargyl-Puromycin (OPP) for 30 min at 37 ° C followed by staining with 5 FAM Azide using the Protein Synthesis Assay kit (Cayman Chemical). The cells were analyzed by Flow cytometry (FACSVerse; BD). Representative histograms for the levels of translating polypeptides (FAM) are shown (Left panel). Mean Fluorescence Intensities from the experiments were shown in the right panel. $n = 3$ biologically independent samples. Individual points and means \pm s.d. are shown.



Supplementary Figure 4. N4BP1 deficiency does not affect the expression of a set of house keeping genes, genes involved in cell cycling, apoptosis, IFN responses, IL17A and IL22 in N4BP1 KO Jurkat cells.

(a) Total RNA was prepared from control and N4BP1 KO Jurkat cells cultured for 1 day, and expression levels of the indicated genes including house-keeping genes (*GAPDH*, *ACTB* or *HPRT*), the anti-apoptotic gene *BCL2*, cell cycle-related genes (*MYC*, *CDK9*, *CCNT1*, *CDK2* and *BRD4*), *IFNB* and inflammatory and anti-inflammatory genes (*NFKBIA*, *IL17A* and *IL22*) were determined by RT-qPCR. Individual points and means \pm s.d. are shown. Data are from $n = 3$ biologically independent samples.

(b) Cell lysates were prepared from control and N4BP1 KO Jurkat cells cultured for 1 day, and the expression of N4BP1, GAPDH, Tubulin- α and I κ B α proteins was determined by immunoblot analysis. β -actin was used as loading controls. Data are representative of two independent experiments



Supplementary Figure 5. N4BP1 restricts HIV-1 in Jurkat cells.

(a) N4BP1 expression levels were determined by RT-qPCR in Jurkat cells transfected with N4BP1 specific siRNA for 72 hours. $n = 3$ biological replicates. Individual points and means \pm s.d. are shown.

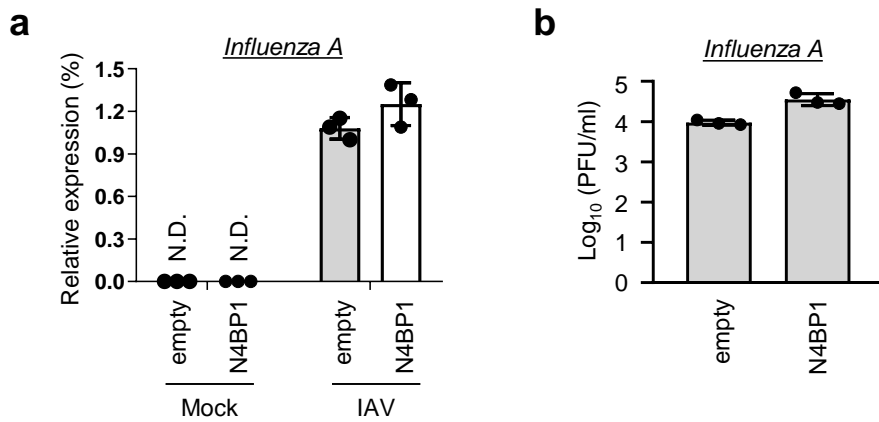
(b and c) siRNA-treated Jurkat cells were infected with HIV-1 NL4-3. Seventy two hours post-infection, *IFNB* and viral mRNA expression levels in the cell lysates (b) and the culture supernatants (c) were determined by qPCR. $n = 3$ biological replicates. Individual points and means \pm s.d. are shown.

(d) Immunoblot analysis for Flag-tagged N4BP1, endogenous N4BP1 and β -Actin in cell lysates from Jurkat cells stably expressing N4BP1.

(e) Expression levels of *tat/rev*, *vif* or *gag* RNA were measured by qPCR in Jurkat cells stably expressing N4BP1 or the respective parental cell line following infection with HIV-1 for the indicated periods. Data are shown as mean values \pm s.d. of technical replicates ($n = 3$).

(f) N4BP1-stably expressing Jurkat cells and control cells were infected with VSV-G-pseudotyped HIV-1 NL4-3 Δ env. Forty eight hours post infection, culture supernatants (Sup) and cell lysates were collected and analyzed by immunoblotting for the expression of Gag p24 in the culture supernatants (Sup), and N4BP1 and β -Actin in cell lysates.

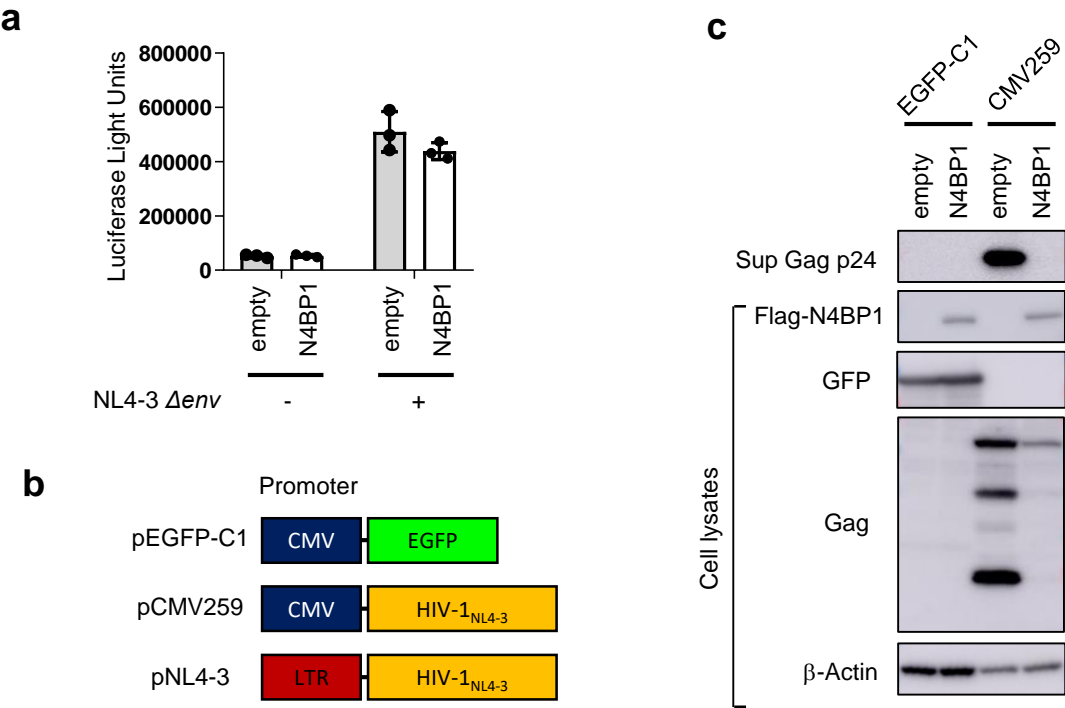
P values were calculated using unpaired two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. n.s. not significant. Data are representative three (a-e) and two (f) independent experiments.



Supplementary Figure 6. N4BP1 does not suppress influenza virus.

(a) HEK293T cells stably expressing N4BP1 or control cells were infected with Influenza A virus (PR8 strain) for 24 hours before the expression level of viral mRNA for segment 4 (HA) was measured by qPCR. N.D., Not detected. $n = 3$ biological replicates. Individual points and means \pm s.d. are shown.

(b) HEK293T cells stably expressing N4BP1 or control cells were infected with Influenza A virus (WSN strain) at an MOI of 1×10^{-3} , and the culture media were harvested 24 hours postinfection. The virus titers were determined by using plaque assays on MDCK cells. Individual points and means \pm s.d. are shown. Data are from $n = 3$ biologically independent samples.



Supplementary Figure 7. N4BP1 inhibits HIV-1 at the post-transcriptional level.

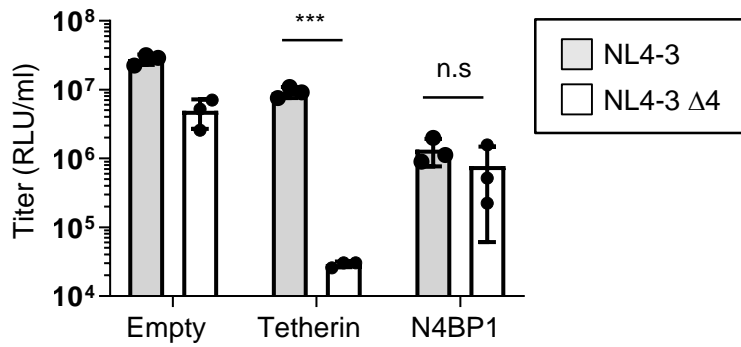
(a) LTR promoter activity was determined in TZM-bl cells cotransfected with N4BP1 and HIV-1 NL4-3 Δenv expressing plasmids. n = 3 biological replicates. Individual points and means \pm s.d. are shown.

(b) Schematic representation of plasmids used in (c). The pEGFPC1 plasmid (Clontech) expresses EGPF protein under the control of CMV promoter. pCMV259 expresses HIV-1 NL4-3 under the CMV promoter instead of LTR (pNL4-3).

(c) HEK293T cells were transfected with pEGFP-C1 or pCMV259 together with or without Flag-N4BP1 plasmids. Expression of Gag p24 in the culture supernatants (Sup) as well as the expression of Flag-N4BP1, GFP, Gag and β -Actin in the cell lysates were determined by immunoblotting 48 hours after transfection. Data are representative of two independent experiments.

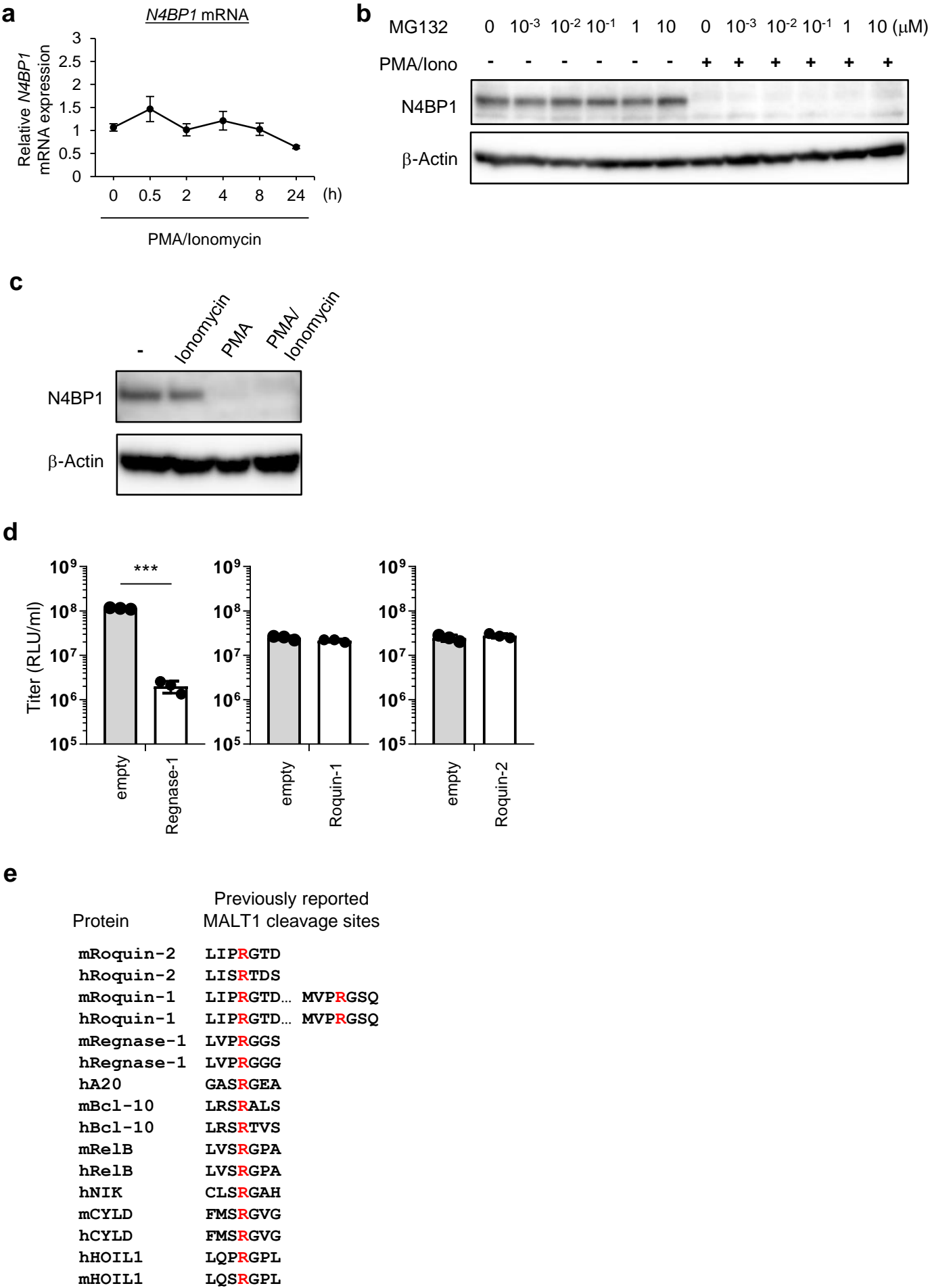
Supplementary Figure 8. N4BP1 inhibits HIV-1 at the late stage depending on its RNase activity.

- (a) Purified N4BP1 was subjected to the *in vitro* cleavage assay using [³²P]-labeled RNA derived from a part of the pBluescript vector.
- (b) Multiple sequence alignment of the N4BP1 and Regnase-1 RNase domains. Arrows indicate conserved Asp residues in the catalytic domain.
- (c) N4BP1 knockdown Jurkat cells exposed to HIV-1 NL4-3 (MOI; 0.1) or heat-inactivated HIV-1 NL4-3 at 37° C for 2 hours. Total DNA was isolated 12 hours after treatment, and amounts of early RT (R/U5) and late RT (U5/gag) products and integrated provirus were quantified by qPCR in. n = 3 biological replicates. Individual points and means ± s.d. are shown.
- (d) HEK293T cells were cotransfected with pNL4-3 and vectors expressing N4BP1 or N4BP1 D623N. Shown are the immunoblots for the expression of Vif, Vpu, Nef and Vpr in cell lysates prepared 48 hours after transfection.
- (e and f) HEK293T cells were transfected with WT or D623N N4BP1 plasmids and cells were counted (e) or analyzed by flow cytometry after propidium iodide staining (f) 48 h later. Data are mean ± s.d. of biological replicates. Data are representative three (c and d) and two (a) independent experiments.



Supplementary Figure 9. N4BP1 is not antagonized by accessory proteins of HIV-1 NL4-3.

HEK293T cells were transfected with an expression plasmid for N4BP1, Tetherin or empty vector together with NL4-3 wild type or NL4-3 Δ4. The latter does not express any of the accessory proteins encoded by HIV-1 (i.e. Vpu, Vif, Vpr and Nef). Infectious virus production was measured by TZM-bl assay 48 hours after transfection = 3 biological replicates. Individual points and means \pm s.d. are shown. *P* values were calculated using unpaired two-tailed Student's *t*-test. ****P* < 0.005. n.s. not significant.



Supplementary Figure 10. N4BP1 is degraded by MALT1 and contribution of other MALT1 substrates in the inhibition of HIV-1.

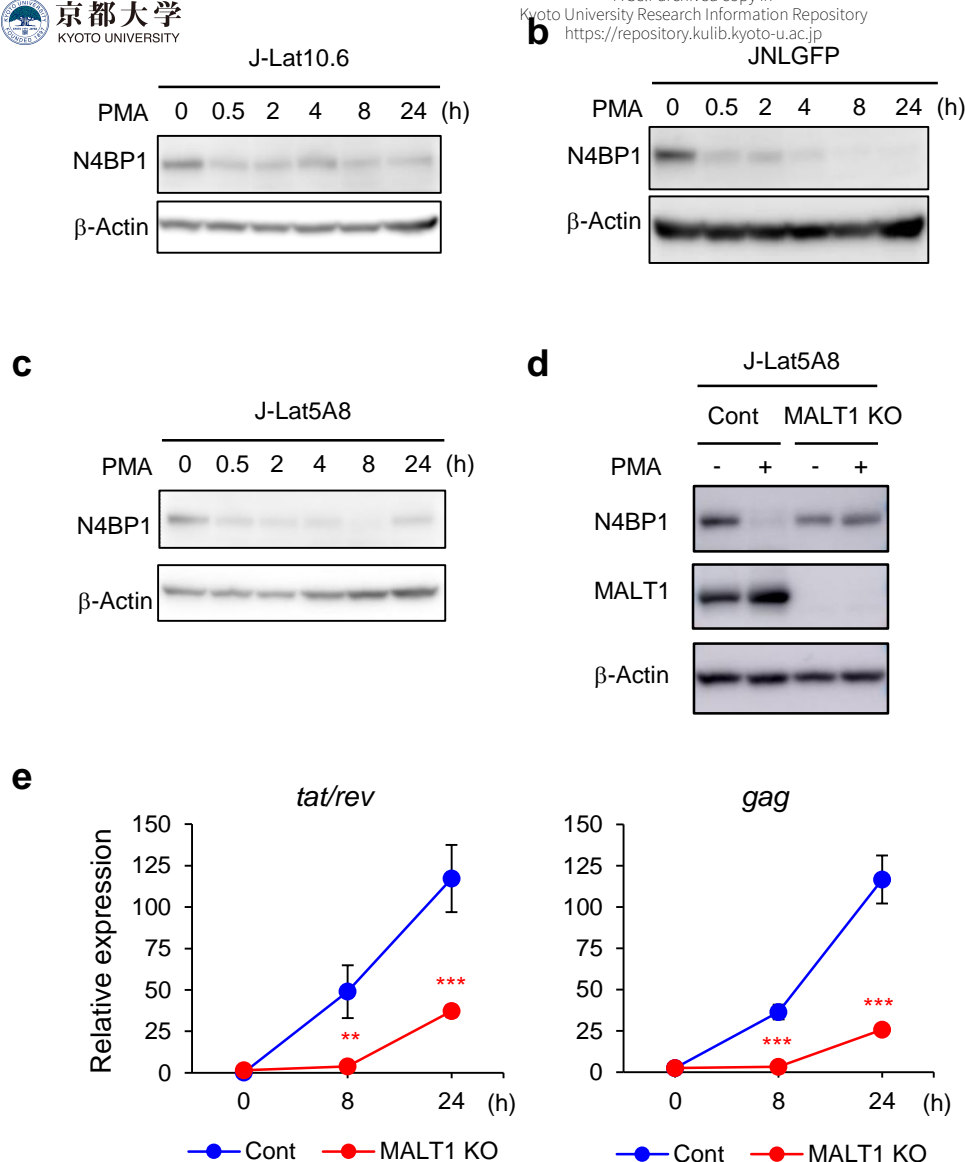
(a) Expression levels of *N4BP1* mRNA were determined by RT-qPCR in Jurkat cells stimulated with PMA (50 ng/ml) plus ionomycin (1 μ M) for the indicated periods of time. Data are shown as mean \pm s.d. of biological replicates (n = 3).

(b) N4BP1 expression levels were determined by immunoblotting in cell lysates from Jurkat cells stimulated with PMA plus ionomycin for 1 hour with or without the indicated concentrations of proteasome inhibitor MG132. β -Actin was used as loading controls. Data are representative of three independent experiments.

(c) Immunoblot analysis of N4BP1 in cell lysates from Jurkat cells stimulated with PMA (50 ng/ml), ionomycin (1 μ M) or PMA plus ionomycin for 1 hour. β -Actin was used as loading controls. Data are representative of three independent experiments.

(d) HEK293T cells were co-transfected with pNL4-3 and the indicated expression plasmids encoding Regnase-1, Roquin-1 and Roquin-2. Forty eight hours post-transfection, a TZM-bl reporter assay was performed to measure the production of infectious virus in the cell culture supernatants. Infectious virus yields normalized to the empty vector control are shown as mean values \pm s.d. derived from of biological replicates (n = 3). *P* values were calculated using unpaired two-tailed Student's *t*-test. ****P* < 0.005.

(e) Previously reported MALT1 substrate proteins and their cleavage sites.

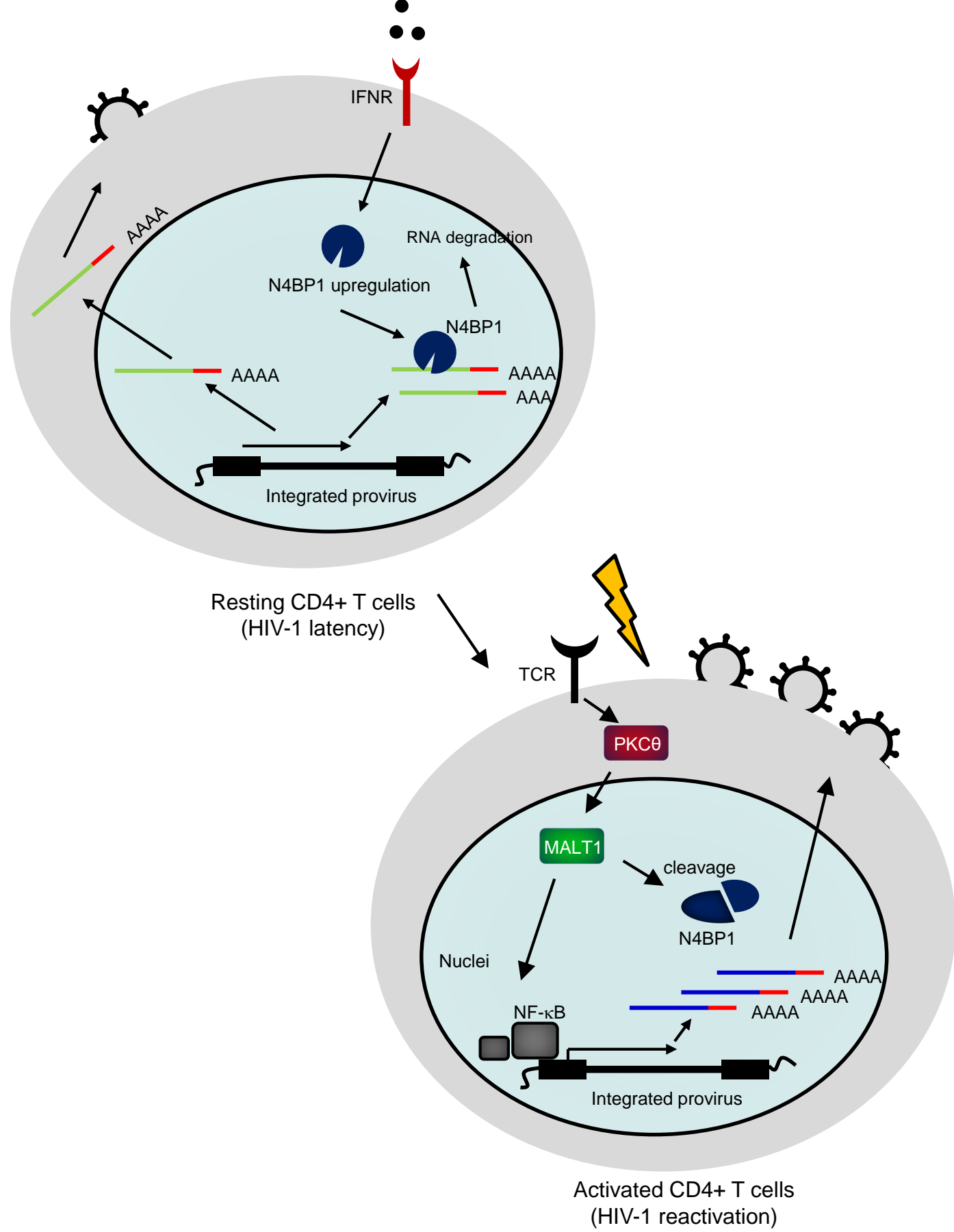


Supplementary Figure 11. MALT1 contributes to viral reactivation in latently infected cells.

(a, b and c) Immunoblot analysis of N4BP1 in cell lysates from J-Lat10.6 (a), JNLGFP (b) or J-Lat5A8 (c) cells stimulated with PMA (50 ng/ml) for the indicated periods of time. β-Actin was used as loading controls. Data are representative of three independent experiments.

(d) Immunoblot analysis of N4BP1 and MALT1 in cell lysates from MALT1-deficient or control J-Lat5A8 cells stimulated with PMA (50 ng/ml) for 24 hours. β-Actin was used as loading controls. Data are representative of three independent experiments.

(e) Expression of *tat/rev* and *gag* mRNAs was quantified by qPCR in MALT1-deficient or control J-Lat5A8 cells stimulated with PMA (50 ng/ml) for the indicated periods. Data are shown as mean ± s.d. of biological replicates (n = 3). *P* values were calculated using unpaired two-tailed Student's *t*-test. ***P* < 0.01; ****P* < 0.005.



Supplementary Figure 12. Schematic model of the function of N4BP1 in inhibiting HIV-1 infection and its regulation in HIV-1 reactivation.

Supplementary Table 1. Genes used for the screening of proteins harboring RNA binding domains and inhibiting HIV-1 infection.

No	Gene Name	Accession Number	No	Gene Name	Accession Number
1	GPATCH4	BC056904	32	ZC3H7A	BC027330
2	GPATCH2	BC042193	33	IREBP1	BC018103
3	GPATCH2	BC063474	34	ZCCHC17	BC050609
4	FTSJD2	BC031890	35	ZC3H8	BC032001
5	PINX1	BC015479	36	HNRNPC	BC103758
6	KIAA0391	BC032221	37	CPSF4	BC050738
7	RBM5	BC046643	38	CPSF4L	BC004603
8	ZCCHC4	BC016914	39	Zc3h15	BC031845
9	NKRF	BC047878	40	Zc3h14	BC024824
10	GPATCH8	BC019948	41	Zc3h11a	BC005786
11	GPATCH4	BC056904	42	ZFP36L1	BC018340
12	GPATCH3	BC096468	43	TIAL1	BC010496
13	SRRD	BC017682	44	Zc3h18	BC030495
14	RBM10	BC004674	45	HNRNPD	BC011172
15	RBM6	BC026129	46	Zcchc6	BC023880
16	ARFRP1	BC010713	47	Zc3h6	BC043311
17	ARFPR1	BC021513	48	HNRNPK	BC006694
18	ZCCHC9	BC032736	49	N4BP1	BC004022
19	PNPT1	BC027228	50	PCBP3	BC042440
20	QKI	BC019917	51	PCBP4	BC010694
21	ANKHD1	BC040231	52	KHDRBS1	BC002051
22	FXR1	BC019139	53	RNF141	BC018104
23	TARDBP	BC071657	54	RNF11	BC020964
24	YTHDC1	BC053863	55	RNF32	BC015416
25	YTHDC1	BC041119	56	PCBP2	BC107688
26	RIG-I	BC015946	57	RNF7	BC008627
27	FUBP1	BC014763	58	RNF138	BC018107
28	ZCCHC7	BC036940	59	RNF6	BC034688
29	ZCCHC11	BC048301	60	IGF2BP3	BC065269
30	ZCCHC18	BC017627,	61	RNF213	BC032220
31	ZC3H7A	BC046363	62	MEX3b	BC111545

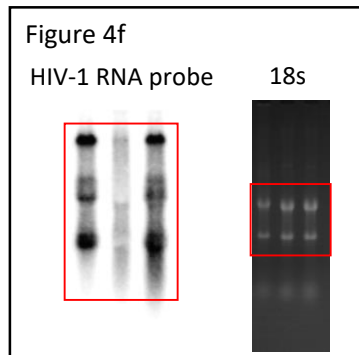
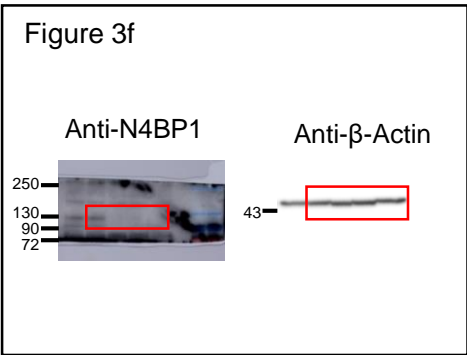
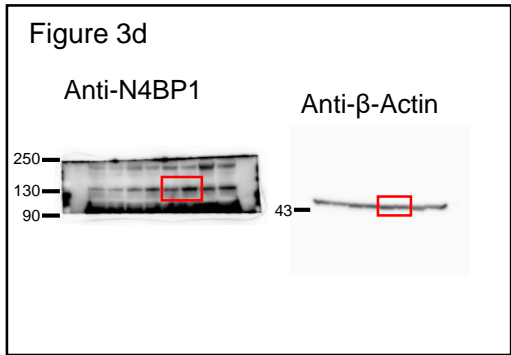
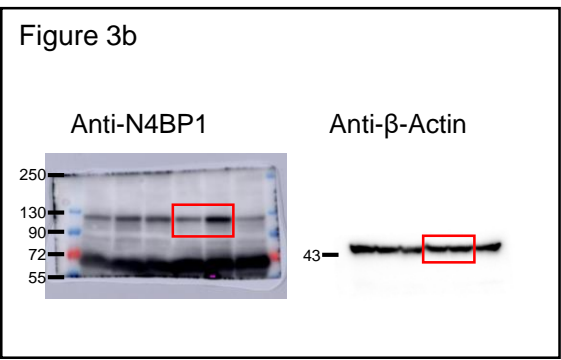
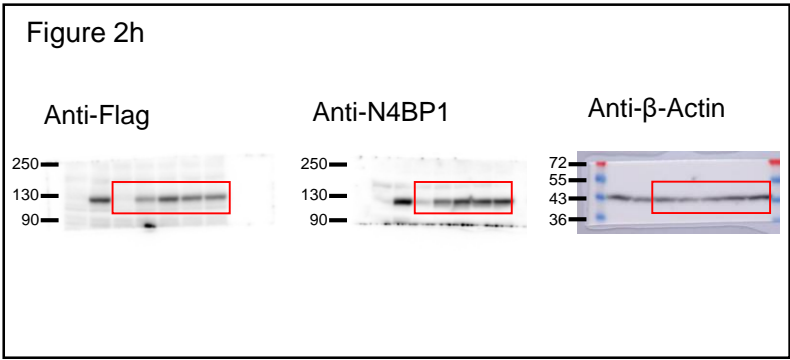
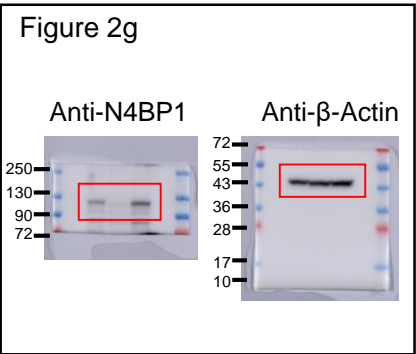
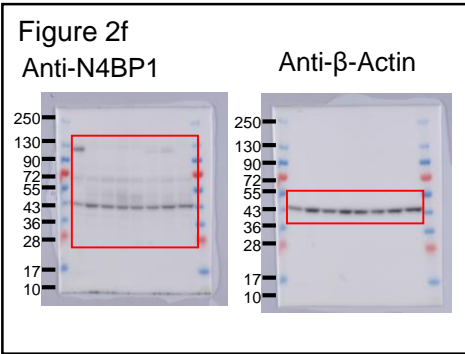
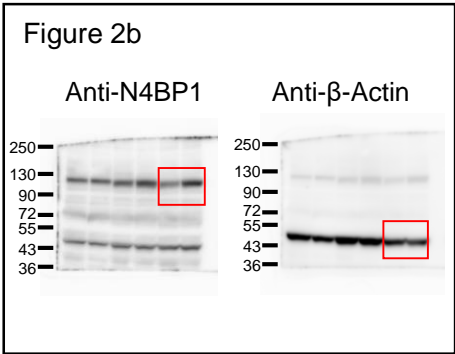
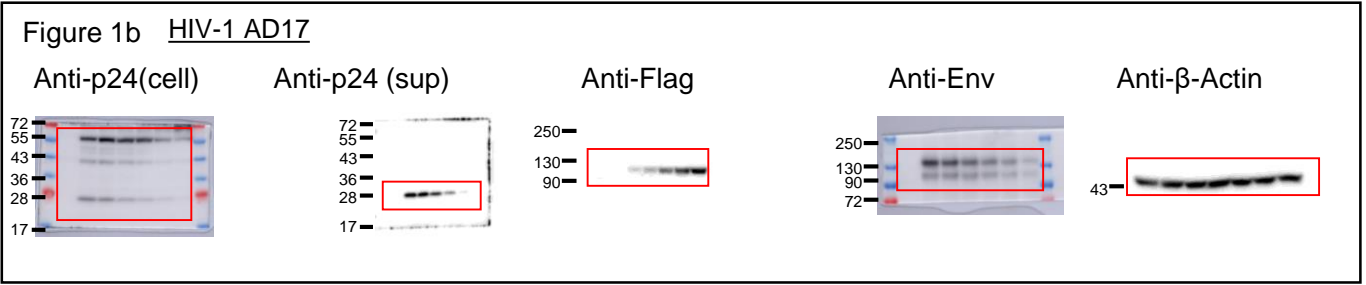
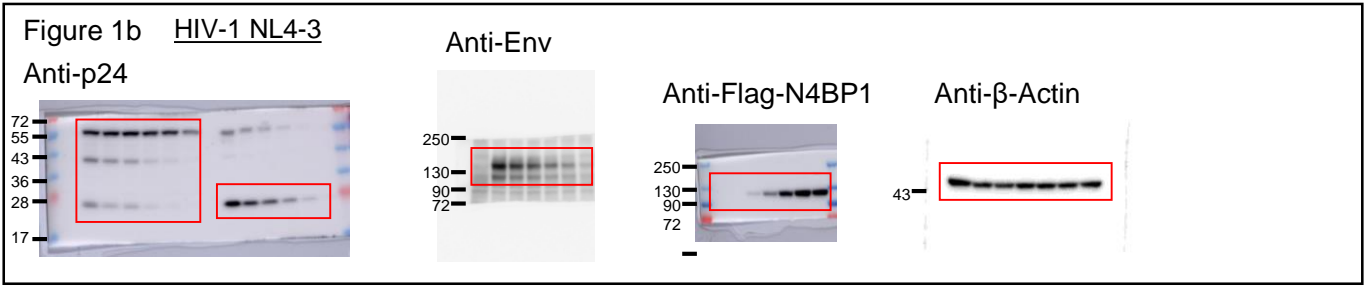
Supplementary Table 2. RT-qPCR Primer Sets

Accession No	Gene	Forward	Reverse
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AF324493.2	Gag2	5'-GTGTGGAATCTCTAGCAGTGG-3'	5'-CGCTCTCGCACCCATCTC-3'
AF324493.2	Gag3	5'-GTGTGGAATCTCTAGCAGTGG-3'	5'-CGCTCTCGCACCCATCTC-3'
AF324493.2	Vif	5'-GGCGACTGGGACAGC-3'	5'-CACACAATCATCACCTGCC-3'
NM_153029.4	N4BP1	5'-CCCGATGATCCTCTGGGAAG-3'	5'-TTTGGCAGGGCACTGAGTAG-3'
NM_004335.4	Tetherin	5'-GTGTCGAATGTCACCCATC-3'	5'-GGGAAGCCATTAGGGCCATC-3'
NM_002176.4	IFN- β	5'-TTGTTGAGAACCTCTGGCT-3'	5'-TGAATATGGTCCAGGCACAG-3'
X03205.1	18S	5'-CGGACAGGATTGACAGATTG-3'	5'-CAAATCGCTCCACCAAGTAA-3'
NM_005101.4	ISG15	5'-ACTCATCTTTGCCAGTACAGGAG-3'	5'-CAGCATCTTCACCGTCAGGTC-3'
NC_002017.1	Influenza A (segment 4; HA)	5'-GGCCCAACCACAACACAACC-3'	5'-AGCCCTCCTTCTCCGTCAGC-3'
AF324493.2	HIV-1 Total	5'-CCTCAGATGCTGCATATAAG-3'	5'-CAGGCTCAGATCTGGTCTAA-3'
NC_001501	MLV gag	5'-GTCTGAGAATATGGGCCAGA-3'	5'-CTTGATCTTAACCTGGGTGA-3'
X52154.1	SIVcpzPtt	5'-ACATCTAGTATGGGCCGAA-3'	5'-TTCTGGAGAACCAATGTCTACC-3'
Y07723.1	HFV gag	5'-ACTTGATGTTGAAGCTCTGG-3'	5'-ATCCTTCAGTAAGGCGAAGA-3'
NM_001256799.2 NM_001289745.2 NM_001289746.1 NM_001357943.1 NM_002046.7	GAPDH	5'-GTTGCCATCAATGACCCCTCATTGACC-3'	5'-CAGCATCGCCCCACTTGATTTTGG-3'
NM_001101.5	ACTB	5'-GCGAGAAGATGACCCAGATC-3'	5'-CCAGTGGTACGGCCAGAGG-3'
NM_000194.3	HPRT1	5'-TATGGCGACCCGAGCCCT-3'	5'-CATCTCGAGCAAGACGTTTCAG-3'
NM_000633.2 NM_000657.2	BCL2	5'-TCCCTCGCTGCACAAATACTC-3'	5'-ACGACCCGATGGCCATAGA-3'
NM_001354870.1 NM_002467.6	cMYC	5'-GCGTCCTGGGAAGGGAGATCCGGAGC-3'	5'-TTGAGGGGCATCGTCGCGGGAGGC TG-3'
NM_001261.3	CDK9	5'-ATGGCAAAGCAGTACGACTCG-3'	5'-GCAAGGCTGTAATGGGGAAC-3'
NM_001240.4	CCNT1	5'-ACAACAAACGGTGGTATTTCACT-3'	5'-CCTGCTGGCGATAAGAAAGTT-3'

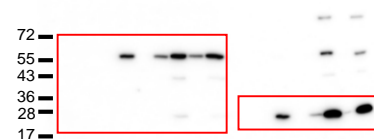
NM_001277842.1			
NM_001290230.1 NM_001798.5 NM_052827.3	CDK2	5'- TTTGCTGAGATGGTGACTION -3'	5'- CTCATCCAGGGGAGGTACA -3'
NM_001330384.1 NM_014299.2 NM_058243.2	BDR4	5'- ACCTCCAACCCTAACAAGCC -3'	5'- TTTCCATAGTGTCTTGAGCACC -3'
NM_020529.2	NFKBIA	5'- GAGGAGTACGAGCAGATGGTC -3'	5'- CAGGTTGTTCTGGAAGTTGAG -3'
NM_002190.3	IL17A	5'- CAACCGATCCACCTCACCTT -3'	5'- GGCACTTTGCCTCCCAGAT -3'
NM_020525.5	IL22	5'- GCAGGCTTGACAAGTCCAACCT -3'	5'- GCCTCCTTAGCCAGCATGAA -3'

Supplementary Table 3. Sequence used for *in vitro* transcription and *in vitro* cleavage assay (Fig. 4d).

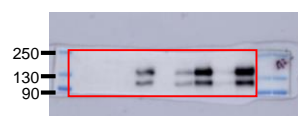
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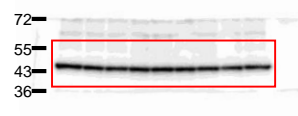
Anti-p24



Anti-Env



Anti-β-Actin



Anti-Flag



Figure 4g

Anti-Flag (IP) Anti-Flag (Input) Anti-β-Actin

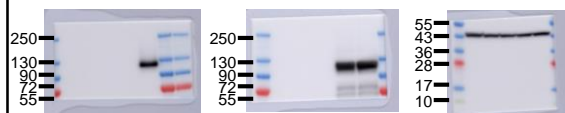


Figure 5a

Anti-N4BP1 Anti-β-Actin

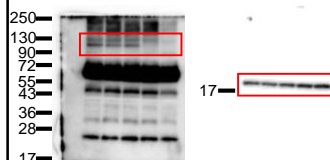


Figure 5b

Anti-N4BP1 Anti-β-Actin

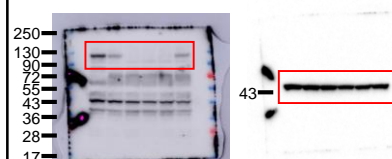


Figure 5c

Anti-N4BP1 Anti-MALT1 Anti-β-Actin

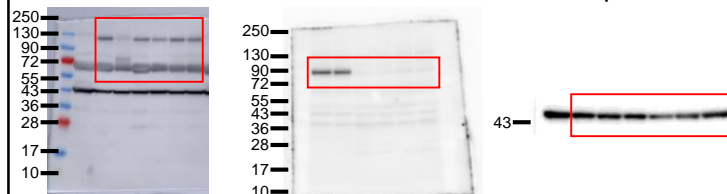


Figure 5d

Anti-N4BP1 Anti-β-Actin

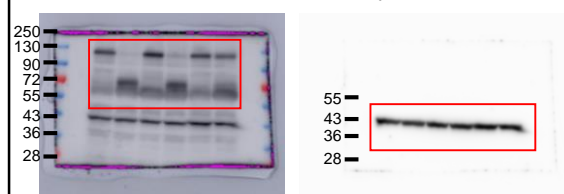


Figure 5f

Anti-Flag

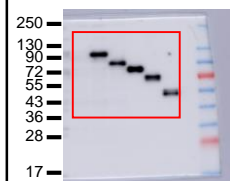
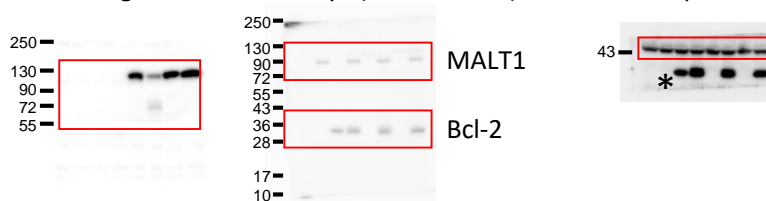


Figure 5i

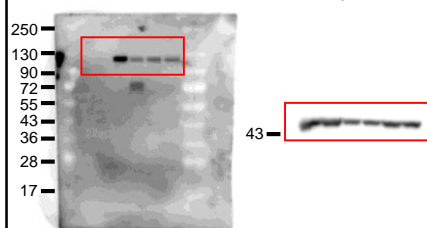
Anti-Flag Anti-Myc (Bcl-2 MALT1) Anti-β-Actin

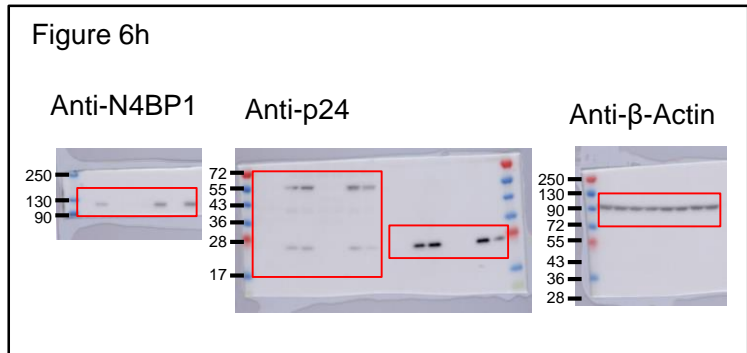
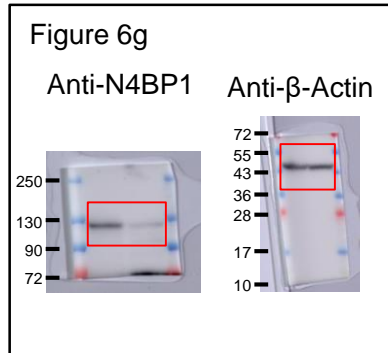
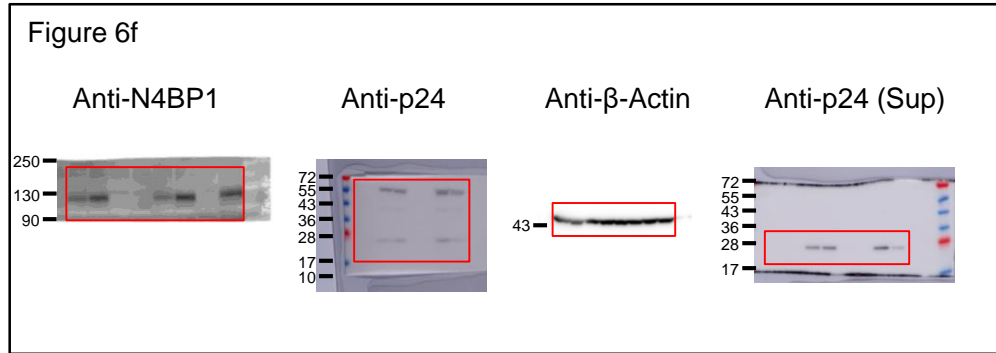
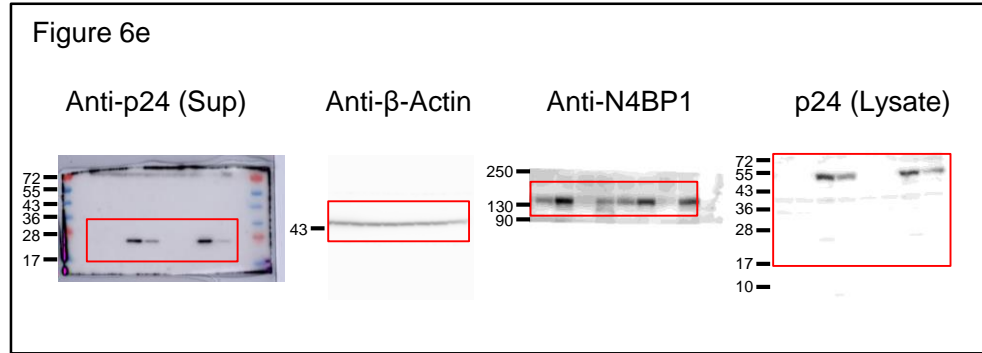
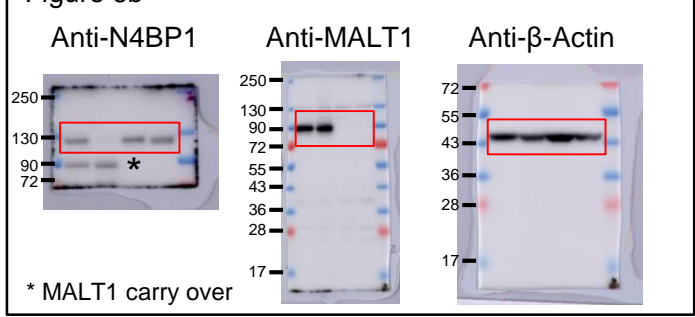
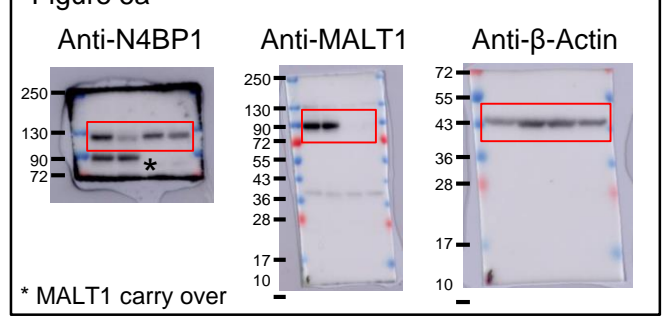


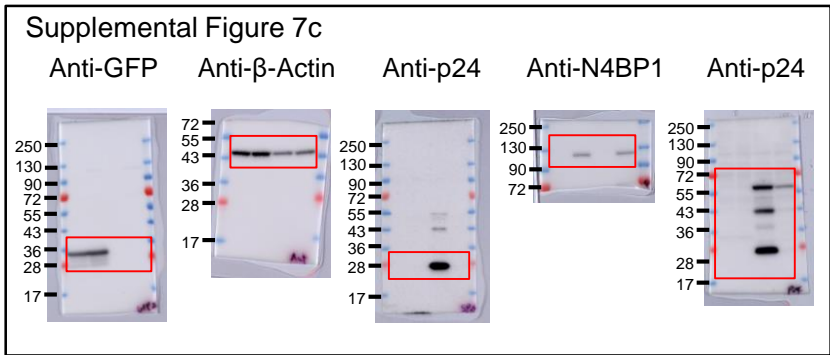
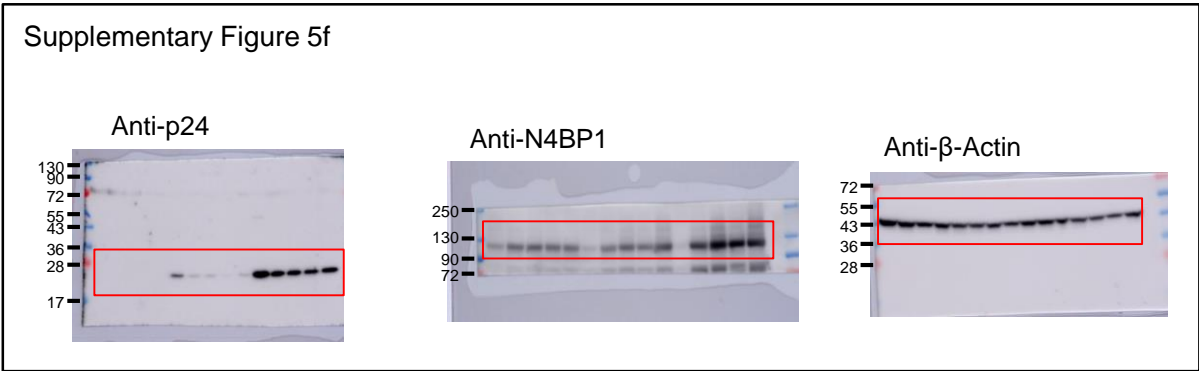
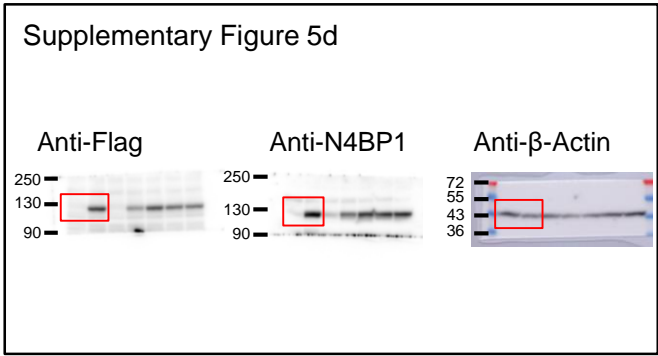
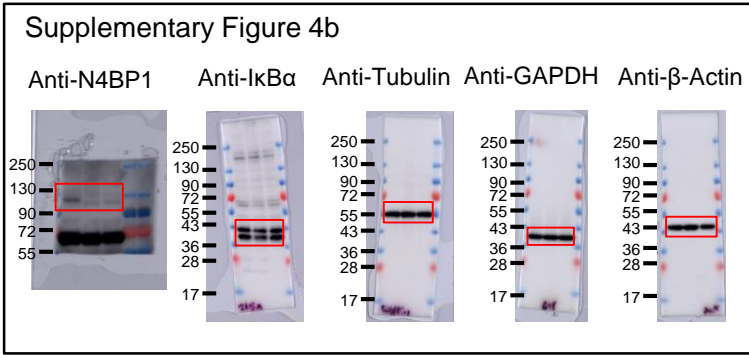
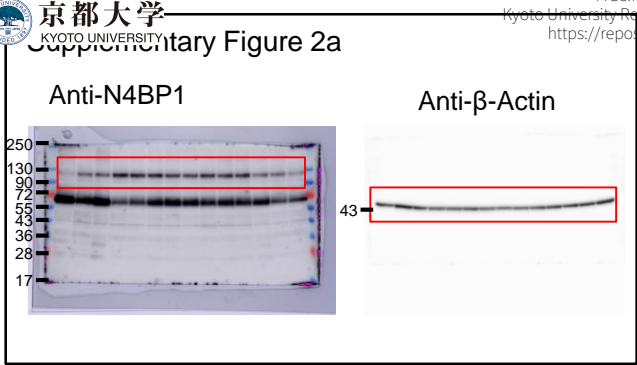
* Bcl-2 carry over

Figure 5j

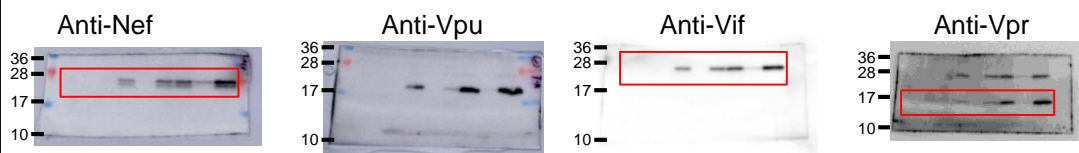
Anti-N4BP1 Anti-β-Actin



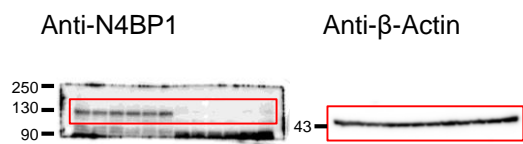




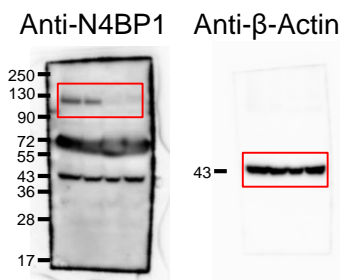
Supplemental Figure 8d



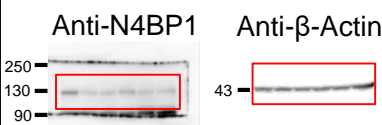
Supplemental Figure 10b



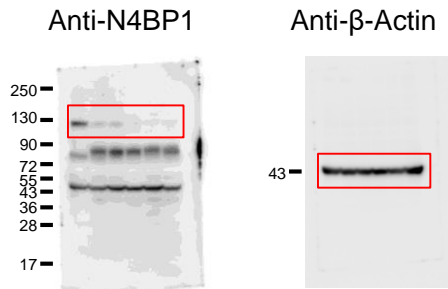
Supplemental Figure 10c



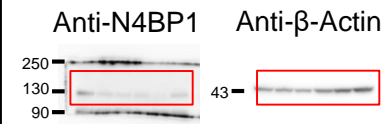
Supplemental Figure 11a



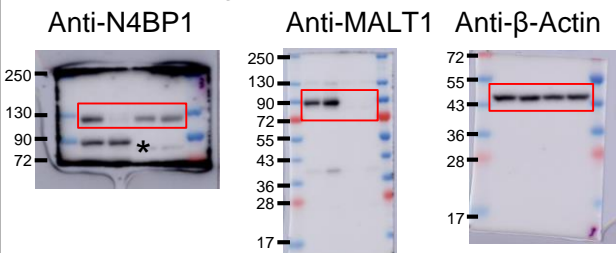
Supplemental Figure 11b



Supplemental Figure 11c



Supplemental Figure 11d



* Bcl-2 carry over